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ON THE ACTION OF L-ASCORBIC ACID
I. ANTIKETOGENIC ACTION OF L-ASCORBIC ACID

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(Received for publication, November 30, 1950)

On a subject of diabetes mellitus, which was insensitive to insuline, acetone bodies were strongly proved by Legal's reaction in his urine. By the administration of insuline they did not disappear in the urine. During the period of observation, the diet was unchanged. So the writer has tried to increase carbohydrate in the diet. Glucose and L-ascorbic acid have the same carbon atoms and both are reducing substances. So it is considered that it should be possible to replace L-ascorbic acid with glucose. For this reason the writer has made the effort to find the evidence for an effect of L-ascorbic acid upon the oxidation of fatty acids. Daily 100 mg. of L-ascorbic acid were subcutaneously injected under the administration of insuline. The results were striking, and the writer recognized that the acetone bodies in urine were not only reduced but also disappeared, presumably due to the action of L-ascorbic acid.

In short, it has long been known that in the condition of fast or omission of carbohydrate from diet in man for a few days, acetone appears in breath, and acetone, acetoacetic, and β-hydroxybutyric acids are excreted in urine. Stadelmann demonstrated that the same substances were excreted in larger amounts in cases of severe diabetes and were responsible for the acidosis coming in this disease.

The precursos of the acetone bodies are known to be chiefly fats and some of the amino acids of protein, such as phenylalanine and tyrosine. The fact, first pointed out by Hirschfeld (*1*) in 1895, and well confirmed later, that they appeared when the amount of carbohydrate catabolized was small, may demonstrate a relationship between carbohydrate metabolism and the production or the non-production of the acetone bodies. Shaffer (*2*) showed that glucose and related substances such as fructose and glycerol exerted a marked effect upon the oxidation of acetoacetic acid by hydrogen peroxide in alkaline solution. At room temperature or in the incubator, and in the absence

of glucose, alkali salts of acetoacetic acid are only very slowly oxidized by hydrogen peroxide, but it glucose is present the disappearance of the keto-acid is rapid, the rate of which being increased with the rise of temperature, the increasing alkalinity of the solution and the increasing amounts of glucose. Fat is not decomposed into carbon dioxide and water, but remains in acetoacetic and β-hydroxybutyric acid—complete oxidation of fat needs simultaneously the oxidation of glucose. As an illustration, Rosenfeld (*3*) explained this relationship as follows: "Fat is burned in the fire of glucose."

Shaffer concluded that one molecule of acetoacetic acid was produced from one molecule of fatty acid, and two molecules of aceto-acetic acid were completely oxidized in the presence of one molecule of glucose. This fact has been recognized as ketolytic theory of glucose for the last twenty years in the field of physiology.

In the present paper the writer will present the experiments which illustrate the ketolytic action of L-ascorbic acid, and which bring out the effect of alkalinity and temperature upon the reaction. A discussion of the reaction products and its chemical mechanism will be postponed to a later paper.

In the following paper the writer attempts to apply the conception that L-ascorbic acid will be replaced with glucose.

EXPERIMENTAL

Acetoacetic acid was prepared from the ethyl ester by saponification with 1 *N* sodium hydroxide at room temperature for 24 hours. The solution was acidified with sulfuric acid using congo red as an indicator, and thrice extracted with a half volume of ether in a separatory funnel. The ether layer was separated, shaken in the second separatory funnel with a small volume of water containing a few drops of alizarin red, and kept alkaline by the addition of NaOH. The keto-acid was quickly and completely absorbed as sodium salt by the water, and the same ether was thus used repeatedly for extraction of the saponified ester solution. The alkaline solution of the salt was aerated with a strong air current for 10 minutes in order to remove acetone, the filtered, and kept in an ice box.

When the solutions were boiled, preferably in acid solution, the keto-acid was rapidly and quantiatively decomposed into acetone which could be determined in the distillate by the iodine titration.

In alkaline solution hydrogen peroxide alone oxidizes acetoacetic

acid very slowly at ordinary temperatures. At boiling temperature, the oxidation is more rapid during distillation, but the rapid decomposition of the acid into acetone prevents great loss, because acetone is quite resistant to peroxide and quickly distills off. Oxidation during distillation may be wholly avoided by acidifying the mixture before distillation, because neither acetoacetic acid nor acetone is attacked by hydrogen peroxide, and the yield of acetone is quantiative.

The procedure usually followed in the experiments as described below is to mix the known amounts of sodium acetoacetate in slightly alkaline solution with sodium hydroxide, hydrogen peroxide, glucose, and L-ascorbic acid, some one or mare of the components being omitted in various controls.

All the mixtures were diluted to the same volume (usually 40 ml.), the flasks stoppered and placed at about the same temperature for a constant time.

In these experiments the solutions were allowed to stand at room temperature or in the incubator, and aliquot portions were withdrawn from time to time, to which 2 ml. of 1.0 per cent acetic acid 40 ml. of redistilled water were added and the remaining acetoacetic acid, together with a small amount of acetone formed by the slow ketone decomposition, was determined as acetone after distillation. The reaction did not take place in acid solution.

Acetone was collected into a flask to which were added 2 ml. of sodium hydroxide solution ane 5 ml. of $N/200$ iodine solution. Iodform was formed from acetone with iodine. Then, to this flask 2 ml. of 25 per cent sulfuric acid were added. Residual iodine was titrated with $N/200$ $Na_2S_2O_3$ solution. Acetoacetic acid was calculated as acetone by the following formula :

$$\{N/200\ I_2\ (ml.) - N/200\ Na_2S_2O_3\ (ml.)\} \times 0.11 \times \frac{100}{\text{amount used}} = x\ mg./dl.$$

(1 ml. of $N/200$ $Na_2S_2O_3$ solution is titrated with 0.11 mg. of acetoacetic acid as acetone.)

RESULTS

There was little, if any, disappearance of acetoacetic acid on standing at room temperature with L-ascorbic acid (in the absence of peroxide) in either neutral or alkaline solution. In the presence of hydrogen peroxide in alkaline solution, 4.1 per cent of the acetoacetic

TABLE I

Effect of L-*Ascorbic Acid and Alkali on the Oxidation of Acetoacetic Acid by Hydrogen Peroxide*

Solution No.	Na aceto-acetate 0.003 *M*	L-Ascorbic acid 0.001 *M*	NaOH 0.001 *N*	H_2O_2 *M*/50	(Acetoacetic acid as acetone) decomposed in distillation		
						Difference	per cent Decomposition
	ml.	*ml.*	*ml.*	*ml.*	*mg./dl.*	*mg.*	*per cent*
I	10	10	10	0	25.6		
II	10	0	10	0	25.6		No change
III	10	0	10	10	24.5	1.1	4.2
IV	10	10	10	10	4.46	21.14	82.5

at 15°

acid was oxidized. In the presence of L-ascorbic acid 82.5 per cent of the acetoacetic acid disappeared.

TABLE II

Effect of L-*Ascorbic Acid and Glucose on the Oxidation of Acetoacetic Acid by* H_2O_2

Solution no.	Na aceto-acetate 0.003 *M*	L-Ascorbic acid 0.001 *M*	Glucose 0.001 *M*	Acetoacetic acid (as acetone)		
					Difference	Decomposition
	ml.	*ml.*	*ml.*	*mg./dl.*	*mg.*	*per cent*
I	10	0	0	17.49		
II	10	10	0	1.76	15.73	89.9
III	10	0	10	16.38	1.11	6.3
IV	10	5	5	4.40	13.09	74.8

at 15°

Ten ml. of an approximately 0.003 *M* sodium acetoacetate solution were placed in each of four flasks, to which were added glucose and L-ascorbic acid solutions as stated in the Tables. After 2 hours at room temperature, 2 ml. portions were diluted to 40 ml., and distilled after adding 2 ml. of 10 per cent acetic acid.

The results were as follows: Shaffer showed that acetoacetic acid more rapidly disappeared in the presence of glucose. But as shown in

the above Table, L-ascorbic acid is more effective in respect to ketolytic action than glucose. 89.9 per cent of acetoacetic acid disappears in the presence of L-ascorbic acid. In the presence of glucose the disappearance of acetoacetic acid is only 6.3 per cent. As shown in the solution no. IV in Table II, L-ascorbic acid is more powerful than the mixture of L-afcorbic acid and glucose in regards to ketolytic action.

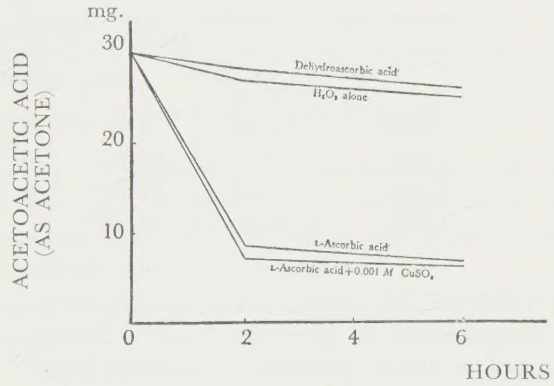

FIG. 1. Effect of L-ascorbic and dehydroascorbic acid on the oxidation of acetoacetic acid by H_2O_2

It is clear that acetoacetic acid is quickly oxidized in the presence of L-ascorbic acid as shown in Table II. The oxidation is promoted by L-ascorbic acid, but not by dehydroascorbic acid. In the presence of dehydroascorbic acid, acetoacetic acid is more slowly oxidized than control as shown in the Fig. 1. Acetoacetic acid is more rapidly oxidized in the presence of Cu^{++} (1 ml. of 0.001 *M* $CuSO_4$), but rather little in the presence of inorganic Fe^{++} (1 ml. of 0.001 *M* $FeSO_4$).

TABLE III

Effect of Alklinity on the Oxidation of Acetoacetic Acid by H_2O_2

Solution no.	Alkalinity as NaOH *N*	Acetoacetic acid as acetone		
			Difference	Decomposition
	Normal	*mg./dl.*	*mg.*	*per cent*
I	±Neutral	22.6		
II	0.0001	18.9	3.7	16.3
III	0.001	8.41	14.19	62.7
IV	0.01	7.85	14.75	65.2

at 15°

This experiment showed a marked increase in the ketolytic action of L-ascorbic acid with the increase of alkalinity of the mixture.

TABLE IV

Effect of L-*Ascorbic Acid on the Oxidation of Acetoacetic Acid by* H_2O_2

Solution no.	L-Ascorbic acid	Acetoacetic acid (as acetone)		
			Difference	Decomposition
	M	*mg./dl.*	*mg.*	*per cent*
I	0	26. 84		
II	0. 0008	20. 90	5. 94	22
III	0. 001	4. 4	22. 44	83
IV	0. 002	1. 21	25. 63	95
V	0. 003	26. 92	−0. 08	

at 15°

As shown in Table IV, the greater is the amount of L-ascorbic acid, the faster is the disappearance of acetoacetic acid. But the oxidation is shown to be inhibited by larger amounts of L-ascorbic acid.

The results of this experiment are plotted in Fig. 2, which shows graphically the relative rate of the decomposition of acetoacetic acid under these conditions, in the presence and absence of L-ascorbic acid.

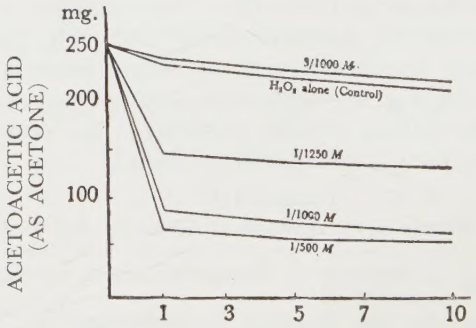

FIG. 2, (at 15°)

As shown in Fig. 2, the rate of ketolytic action is increased with the amount of L-ascorbic acid, but this oxidation is inhibited by larger amount of L-ascorbic acid—that is, when the amounts of L-ascorbic acid exceed a definite molar concentration the ketolytic action does not take place, but the oxidation is later than control.

SUMMARY

1. The oxidation of L-ascorbic acid in alkaline solution by hydrogen peroxide accomplished the disappearance of acetoacetic acid, if the latter be present in the solution. Acetoacetic acid was oxidized very slowly by hydrogen peroxide in the absence of L-ascorbic acid, but the disappearance of acetoacetic acid was rapid even at room temperature when L-ascorbic acid was simulataneously oxidized.

2. The oxidation was promoted by L-ascorbic acid, but not by dehydroascorbic acid.

3. It has been inferred that L-ascorbic acid participates the oxidation in organism.

4. The rate of ketolytic action was increased with alkalinity, as well as with the amount of L-ascorbic acid, especially in the presence of Cu^{+}. But the oxidation was inhibited when the amounts of L-ascorbic acid exceeded a definite molar concentration.

5. An illustration by Rosenfeld. "Acetoacetsic acid is burned in the fire of glucose" will be applicable to this case: "Acetoacetic acid is burned in the fire of L-ascorbic acid."

Sincere thanks are due to Prof. K. Kakinuma for his criticisms and advice, and to Assistant Prof. T. Takahashi for his contant simulating help.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF ACETIC ACID IN THE URINE

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(Received for publication, October 5, 1951)

In 1949, Hutchens and Kass (*1*) reported a colorimetric method for the determination of acetic acid produced in the culture medium of microorganisms. They used lanthanum nitrate which has long been known as the specific reagent for qualititative identification of acetic acid.

This colorimetric procedure was found to be applicable to the urine after distillating it by Friedemann's method (*1*). The distillate must be evaporated to a small volume prior to the colorimetric procedure. Barium hydroxide was found to be the most convenient base among those used during the evaporation.

Reagents:—(a) 10 per cent sodium tungstate solution; (b) 10 *N* Sulfuric acid; (c) Magnesium sulfate. (Epsom salt): (d) Mercuric sulfate—dissolve 100 g. of $HgSO_4$ in 1 liter of 2 *N* H_2SO_4: (e) The saturated barium hydroxide solution: (f) 0.02 *N* Iodine solution—dissolve 2.54 g. of iodine and 33.2 g. of potassium iodide in distilled water and dilute to 1 liter: (g) 0.2 *N* $La(NO_3)_3$ solution—dissolve 2.286 g. of $La(NO_3)_3$ in 100 ml. of distilled water: (h) 0.2 *N* NH_4OH solution.

Apparatus—Two sets of distillation apparatus of Friedeman are used.

Procedure—To 25 ml. of urine in a steam distillation flask are added 4 ml. of 10 *N* H_2SO_4 plus enough water to bring the volume to 30 ml, 15 ml. of tungstate solution are added and mixed thoroughly. Furthermore 8 g. of Epsom salt are added and again mixed by shaking. Steam distillation is carried out. About 200 ml. of the distillate are placed in the second distillation flask, to which 2 ml. of 10 *N* H_2SO_4, 50 g. of Epsom salt, 10 ml. of $HgSO_4$ solution and talcum are added in the order above indicated. The distillation is then carried out and discontinued as soon as the contents begin to crystallize. About 190–200 ml.

of distillate are placed in a evaporating dish, made strongly alkaline by adding about 10 ml. of saturated barium hydroxide solution, and concentrated on a boiling water-bath to a small volume (5 ml.). The excessive $Ba(OH)_2$ are removed by bubbling CO_2. The residue is filtered in a graduated cylinder. The evaporating dish is washed several times with a small amount of hot water and the washed solution is filtered into the same cylinder. To the combined filtrate is added a small amount of saturated $Ba(OH)_2$ to bring pH at 9.0 (thymolphthalein) and further added a proper amount of distilled water to make the volume up to 15 ml. The solution is then filtered again.

Into a small test tube is put in the following order: (a) 1 ml. of the filtrate containing 100–400 γ of acetic acid; (b) 1 ml. of 0.02 N iodine solution; (c) 0.5 ml. of 0.2 N $La(NO_3)_3$ solution, and (d) 0.5 ml. of 0.2 N NH_4OH solution.

The tubes are mixed thoroughly, heated in a boiling water bath for 5 minutes, and cooled with tap water. To the tubes is added enough water to make the volume to 6 ml. The extinction of the green colored solution is measured by Pulfrich's photometer with the filter of S_{66}.

Calibration Curve—For calibration 0.1 to 0.4 mg. of acetic acid are distilled by this method and measured by photometer. The calibration curve is shown in Fig. 1.

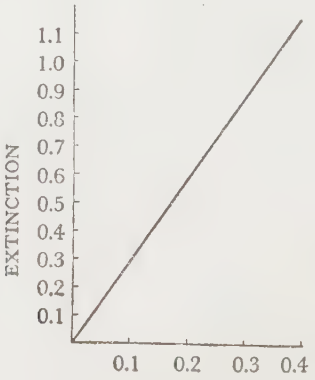

Fig. 1. Standard curve (Filter S_{66}.)

Normal human urine contains 1–2 mg/dl. of acetic acid. The urine of normal rabbit contains 3–4 mg./dl. of acetic acid. Recovery experiments are made by adding acetic acid to urine. 95–100 per cent of

recovery are obtained by this method. Both identification and determination of acetic acid are carried out by this method.

SUMMARY

A colorimetric method for the determination of acetic acid in urine is described.

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A STUDY OF THE ACTION OF AMYLASE AND SO-CALLED "AMYLOSYNTHEASE" BY THE POTENTIOMETRIC IODINE TITRATION*

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(Received for publication, October 17, 1951)

Since the study of Kuhn (*1*) and Ohlsson (*2*) amylase is divided into the α-(dextrinizing) type and the β-(saccharifying) type. Soluble starch has usually been used as the substrate in the investigation of this enzyme. In 1940 Meyer (*3*) found that starch generally consisted of two different fractions, amylose and amylopectin, the former being a linear unbranched chain compound, and the latter a branched chain compound. Thus it has now become desirable for us to investigate the action of α- and β-amylases on each of these two fractions of starch. Hitherto the methods of estimating the activity of amylase consisted of measuring iodine color reaction or viscosity or reducing power of the reaction mixture. But these methods are not satisfactory for measuring exactly the quantity of the substrate split by the amylase, or that of the product formed, especially in the case of the action of α-amylase.

In the present study the potentiometric measurement of the iodine reaction proposed by Beats, French and Rundle (*4*) was used to measure the quantity of amylose. And the similar potentiometric measurement was applied also to measure the quantity of amylopectin. Using this method and, in addition, estimating the reducing power, the action of α-and β-amylases of the amylose and on the amylopepectin was investigated.

In 1930 Nishimura (*5*) found the autolysate of beer-yeast acted on waxy rice starch and changed its iodine color reaction from red

* This report was presented at the Symposium on Enzyme Chemistry held in Tokyo on October 24, 1949, and at the 22nd Meeting of the Japanese Biochemical Society held in Kanazawa on April 29, 1950.

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to blue, and in this case the reaction mixture became more and more turbid until precipitation occurred. He proved that this reaction was caused by an enzyme of yeast, and stated that this enzyme could also act on glycogen. From the fact that this change of iodine color reaction is opposite to the change which results from the action of amylase, Minagawa (*6*) concluded that this new enzyme acted synthetically and named it "amylosynthease." However, Maruo (*7*) stated that the action of this enzyme was not synthetic, but that it split the glucosidic 1.6-linkage of starch and produced the linear structure of molecules from the branched chain structure of waxy rice starch. Furthermore, Fuwa (*8*), on the basis of x-ray study, claimed that the reaction product had a linear structure as amylose. In spite of these investigations, the whole course of the reaction of this enzyme is not yet known in detail, nor is it clear whether all of the reaction products condsist of the molecules of the same molecular weight or not. Therefore these points are the subject of the present study, and hhe method used was the potentiometric titration method above mentioned.

EXPERIMENTAL

The Separation of Amylose and Amylopectin Fractions of Starch—Amylose was prepared from potato starch according to the Schoch's butanol precipitation method (*9*), and was purified by two successive crystallizations from the boiling butanol-water mixture. Amylopectin was prepared from the Schoch's amylopectin fraction, purified by the method of Tanret and Pascu (*10*).

The purity of the prepared amylose was tested by the potentiometric titration, and the result was almost the same as the result which Rundle (*4*) obtained from Kerr's crystalline amylose. Further, the prepared amylose was split off completely by the action of β-amylase. Thus the puritp of this amylose was thought to be satisfactory. The prepared amylopectin was provep free from any contamination by the reddish purple color of the iodine reaction and by the shape of the potentiometric titration curve which gave no indication of amplose contamination at all.

As the substrate of "amylosynthease" soluble starch was prepared from waxy rice, which showed the same potentiometric titration curve as amylopectin.

The Potentiometric Titration of Amylose and Amylopectin—The method used was almost the same as that of Rundle, Beats and French

(*4*). Instead of hydroiodic acid used in the original method, hydrochloric acid and potassium iodide were used. (The final concentration of KI was 0.05 *N*). The final volume of the titrated solution was 50 ml., and the titration was carried out with 1/1000 *M* iodine solution containing the same concentration of potassium iodide as the titrated solution. During the titration the solution was aerated vigorously (especially when the concentration of amylose or amylopectin was high). For the titration 1/1000 *N* iodine solution was dropped in the amount of 0.2 ml. at 2 minute intervals. (Such long intervals are necessary when the titrated material is a mixture of various kinds of molecules of amylose, but they are not necessary when the titrated material is amylopectin). After each addition of the iodine solution the potential of a platinum electrode inserted into the titrated solution was measured, with a saturated calomel cell used as a reference electrode. For the measurement of potentials a potentiometer of Cambridge unipivot type was employed.

The Measurement of the Reducing Power—The reducing power of the reaction product was estimated by the method of Hagedorn and Jensen (*11*) and was calculated as maltose.

The Preparation of Enzymes—As the materials for preparing α- and β-amylases, human salivr, and ungerminated barley were used, respectively. To purify α-amylase from saliva, Meyer's method (*12*) was followed. Saliva was collected fresh, filtered, and fractionated with acetone and twice with ammonium sulfate successively. To purify β-amylase Caldwell's method (*13*) was adopted. "Amylosynthease" was prepared from the compressed beer yeast by the fractionation with ammonium sulfate according to Miyake (*14*). The purity of these amylase preparations was confirmed by the reducing power and the potentiometric titration curves that could be observed when the acted on amylose.

The Experimental Conditions—The action of amylase on amylose or amylopectin was investigated under the following conditions: 1 ml. of freshly prepared enzyme solution was added to the mixture of 2 ml. of the substrate solution, 2 ml. of 2% NaCl, 2.5 ml. of buffer solution, and 2.5 ml. of redistilled water. To obtain the substrate solution a necessary amount of amylose or amylopectin was completely dispersed in 10 ml. of 5 *N* KOH diluted with water, neutralized by the addition of hydrochloric acid, with methylred as an indicator, and finally diluted to a definite volume. As the buffer solution, *N*/10 acetate buffer at pH 4.6 was used for β-amylase, and *M*/5 phosphate buffer at pH 6.4

for α-amylase. The concentration of the enzyme solution was 3 mg. of dry weight per 100 ml. in case of the observations on the action of β-amylase on amylose, and 0.03 mg. per 100 ml. in case of the action of α-amylose on amylose.

For the potentiometric titration the reaction mixture was treated as follows: 5 ml. of 0.5 *N* NaOH were added to each of the reaction mixtures at regular intervals to stop the action of the enzyme. For the potentiometric titration this reaction mixture was transferred quantitatively to a Michaelis vessel, diluted with water, and just before the beginning of the titration it was adjusted to pH 3 with 0.5 *N* HCl filled up with water to a volume of 45 ml., and finally 5 ml. of 0.5 *N* potassium iodide were added.

The action of "amylosynthease" was investigated under the following conditions: 5 ml. of the solution of "amylosynthease" preparation were mixed with 10 ml. of 1.5% waxy rice starch solution and 5 ml. of 1/10 *M* acetate buffer at pH 6.2 in a glass-stoppered test tube and the mixture was placed in an air-thormostat at 25°. Toluene was added as a preservative. After a definite time 5 ml. of the reaction mixture were transferred to a Michaelis vessel, diluted with water, adjusted to pH 3 with dilute HCl and then the potentiometric titration was carried out.

RESULT

The Avilability of the Potentiometric Titration for the Estimation of the Amounts of Amylase and Amylopectin—The course of the change of potential during the titration of amylose with iodine was investigated with various concentrations of amylose solution. Just as Rundle *et al.* (*4*) stated, the amounts of the iodine solution consumed up to the points of the inflexion of the potentiometric titration curves were proportional to the concentrations of amylose. Thus it was confirmed that this method is useful in the estimation of the amount of amylose during the course of the action of the enzyme. When the titration was carried out at pH 3, it was proved that no influence resulted from the reducing substance such as maltose or glucose which would be produced by the action of amylase.

Next, the course of the increase in the potential during the titration of amylopecin with iodine was investigated with various concentrations of amylopectin solution. As Rundle *et al.* (*4*) stated, the potentials, obtained with the solution of amylopectin in 0.05 *N* KI were lower

than those with 0.05 *N* pure KI through the whole course of the titration. Now in the present study the titration of various concentrations of amylopectin was carried out with 0.001 *N* iodine. As shown in Fig. 1, the lowering of the potentials is almost proportional to the concentration of amylopectin. Potentials obtained by the addition of 12 ml. of *N*/1000 iodine were plotted from this figure, and the relationship between

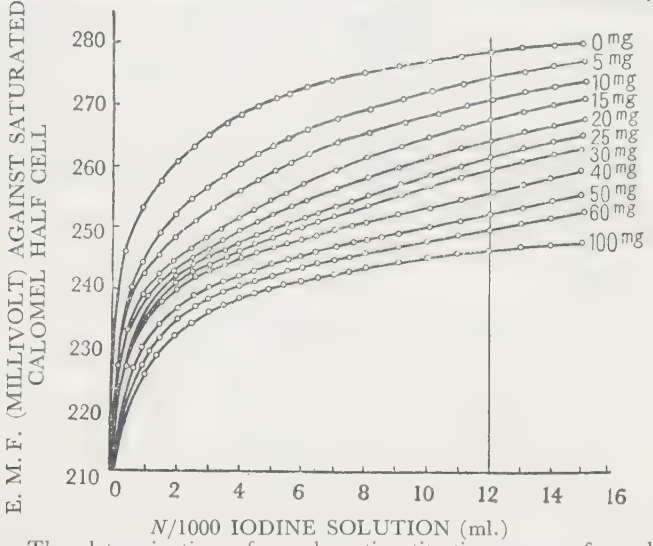


Fig. 1. The determination of amylopectin; titration curves of amylopectin varing in concentrations.

These figures represent the amounts of amylopectin in 50 ml. of the solution.

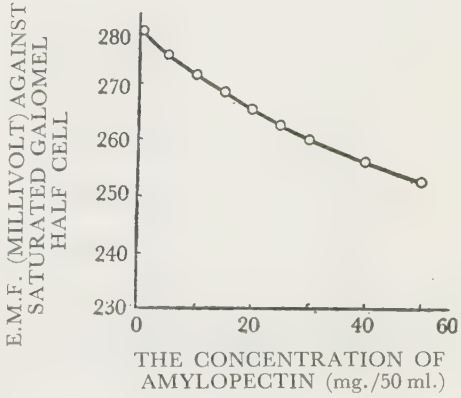


Fig. 2. The determination of amylopectin; the relationship between the concentration of amylopectin (mg./50 ml.) and the potential obtained by the addition of 12 ml. of *N*/1000 iodine.

the concentration of amylopectin and the titration potentials thus obtained is shown in Fig. 2. Using this figure the concentration of amylopectin can be estimated up to the concentration of 0.5 g. in 50 ml. by the potentiometric titration with iodine.

The Action of β-Amylase on Amylose—In this experiment 40 mg./100 ml. and 80 mg./100 ml. solutions of amylose were used as substrate, and β-amylase was added to them as enzyme. Its concentration was 3 mg./100 ml. for the 40 mg./100 ml. amylose solution. The following results were obtained: (a) The blue color of the iodine reaction fainted gradually as the reaction proceeded, but its tint was not changed until the color disappeared; (b) At various reaction times, the reaction mixture was titrated potentiometrically with iodine. As shown in Fig. 3, the potential had a definite value and showed no break from

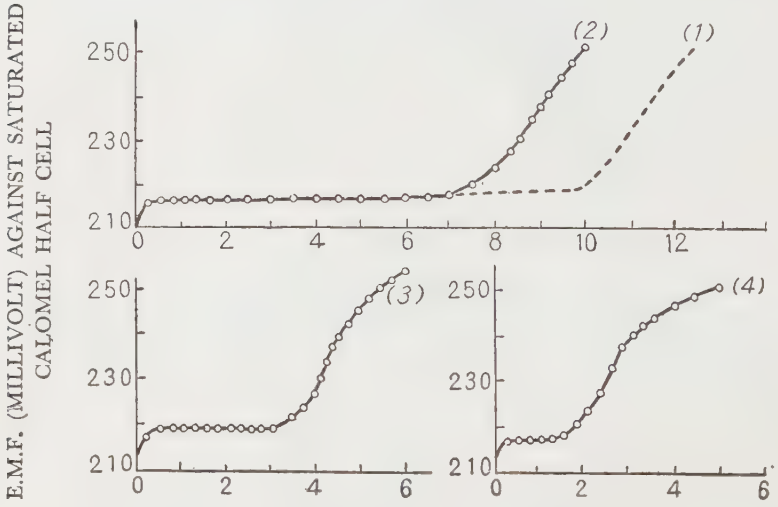


FIG. 3. The action β-amylase on amylose: the iodine titration curves at various reaction times.

(1) Original amylose (control curve) (2) Reaction time: 5 minutes: (3) Reaction time; 20 minutes; (4) Reaction time; 30 minutes.

the beginning up to the neighborhood of the inflexion point. As the reaction proceeded, the amount of the iodine solution required to reach the inflexion point became smaller, but new breaks never appeared. As these findings seemed to indicate that the residual amylose was not

changed in its structure through the course of this reaction, it was confirmed further by the following experiment. Some quantity of the original amylose was added newly to the reaction mixture after some lapse of time, and the total solution was titrated. As shown in Fig. 4,

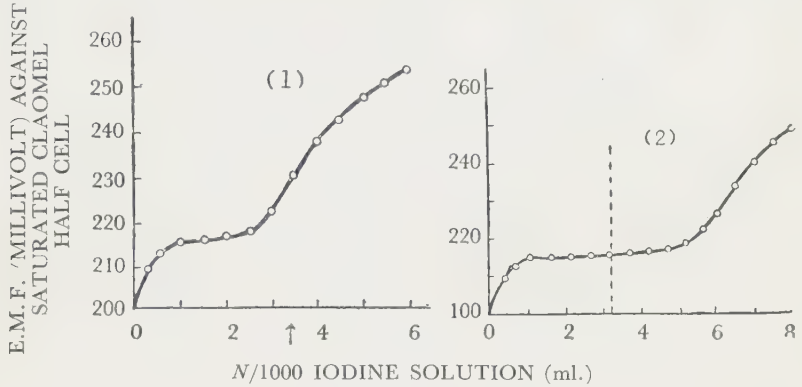


Fig. 4. Action of β-amylase on amylose; the iodine titration curve of the mixture of the reaction product and the original amylose.

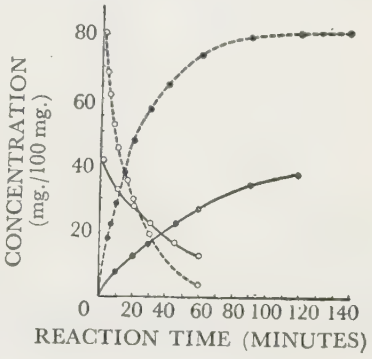
(1) The iodine titration curve of the added amylose (4.4 mg.);

(2) The iodine titration curve of the mixture of reaction product (30 minutes) and the added amylose (4.4 mg.)

the amount of the iodine solution required to reach the inflexion point became larger, but no new breaks occurred; (c) The amount of amylose was estimated by the potentiometoric titration and the maltose produced in the reaction mixture was estimated by the reducing power of the mixture at various points of time. The symmetrical curves were obtained as shown in Fig. 5, for both the concentrations of 40 mg./dl.

Fig. 5. The action of β-amylase on amylose; the relationship between the remaining amylose and the produced maltose.

○ Amylose; ● Maltose. The solid lines indicate the case when the substrate concentration was 41 mg./100 ml.; The broken lines indicate the case when the substrate concentration was 80 mg./100 ml.



and 80 mg./dl. of amylose. The sum of the amounts of the residual amylose and the produced maltose was 90–100% of the amount of the original amylose, as shown in Table I. From this result it is concluded

TABLE I

The Action of β-Amylase on Amylose; the Relationship of the Amounts of Amylose and Maltose and the Reaction Time

The substrate concentration: 80 mg./100 ml.

Reaction time	Amylose	Maltose	The sum of amylose and Maltose
minutes	*mg./100 ml.*	*mg./100 mg.*	*mg./100 ml.*
5	60. 6	18. 0	78. 6
7. 5	51. 5	22. 2	73. 7
10	45. 0	28. 0	73. 0
15	37. 7	35. 4	73. 1
20	29. 3	46. 6	75. 9
30	18. 5	57. 0	75. 5
60	3. 9	76. 5	
75		78. 0	
90		78. 0	
120		80. 6	
150		88. 8	
180		80. 8	

The substrate concentration; 41 mg./100 ml.

Reaction time	Amylose	Maltose	The sum of amylose and Maltose
minutes	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*
10	32. 4	7. 7	40. 1
20	28. 3	12. 6	40. 9
30	22. 8	17. 8	40. 6
45	16. 5	22. 4	38. 9
60	12. 5	27. 9	40. 4
90	——	34. 7	——
120	——	37. 1	——

that there exist only the residual molecules of the original amylose and the molecules of maltose produced by the action of the enzyme. There is no possibility that shorter chain molecules of dextrin are produced

as in the case of the action of α-amylase. When the iodine reaction disappeared, the amount of maltose produced was estimated to be 100% of the substrate; (d) From the course of the decrease in amylose acted by β-amylase it has been concluded that the reaction is a first order reaction, as was already proved from the production of maltose by Kerr (15).

The Action of α-Amylase on Amylose—In this experiment the concentration of the enzyme was 0.03 mg./100 ml., and, as the substrate 80 mg./100 ml. and 110 mg./100 ml. solutions of amylose were used. The same conclusion was obtained by used of both concentrations of amylose. Therefore, the findings on the 80 mg./100 ml. solution given blow were similar to those on the 110 mg./100 ml. solution. (a) The color of the iodine reaction changed, as time went on, from blue to blue purple after 60 minutes, reddish purple after 80 minutes, and then after passing through red orange it disappeared. (b) Several examples of the potentiometric titration curves of this reaction are shown in Fig. 6.

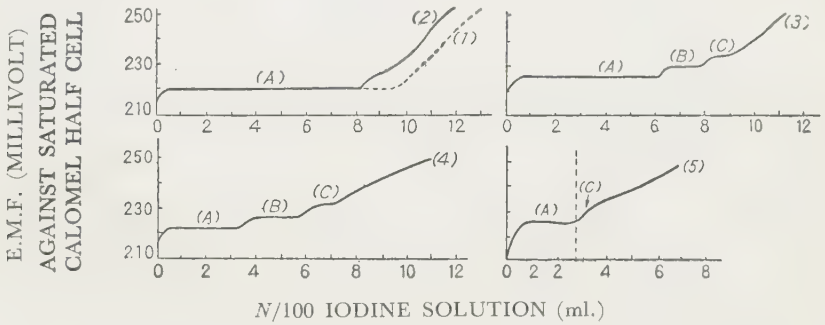


FIG. 6. The action of α-amylase on amylose: the iodine titration curves at various reaction times.

(1) The original amylose; (2) The action time; 5 minutes; (3) The reaction time: 12 minutes; (4) The reaction time: 30 minutes; (5) The reaction time: 75 minutes. 2 mg. of the original aymlose was added to the reaction product. (A) The original amylose; (B) The second amylose; (C) The third amylose

After 5 minutes of the reaction time, the curve corresponding to the original amylose became shorter, and a curve of new amylose began to appear, which was about 4 mv. higher in potential than the original amylose. After 12 minutes the curve of the original amylose became still shorter, and the curve of the second amylose became more distinct,

and at the same time the curve indicating the third amylose appeared, which was about 5 mv. higher in potential than the curve of the second amylose. As time went on, the curve indicating the original amylose became still shorter and the curves indicating the second and the third amylose became longer. The second curve reached the maximum length after 35 minutes, and then it also became shorter. After 75 minutes the original and the second curves disappeared and there remained only the third one; (c) As the length of these curves corresponds to the concentration of amylose, the concentrations of these three sorts of amylose at various reaction times were plotted from these results. The reducing power of the reaction mixture increased very slowly, the value of which was only 12 mg./dl. after 60 minutes, and, after 5 hours, 50 mg./dl. as maltose. The relationship between the amounts of the three sorts of amylose and the reducing power is shown in Fig. 7; (d) To

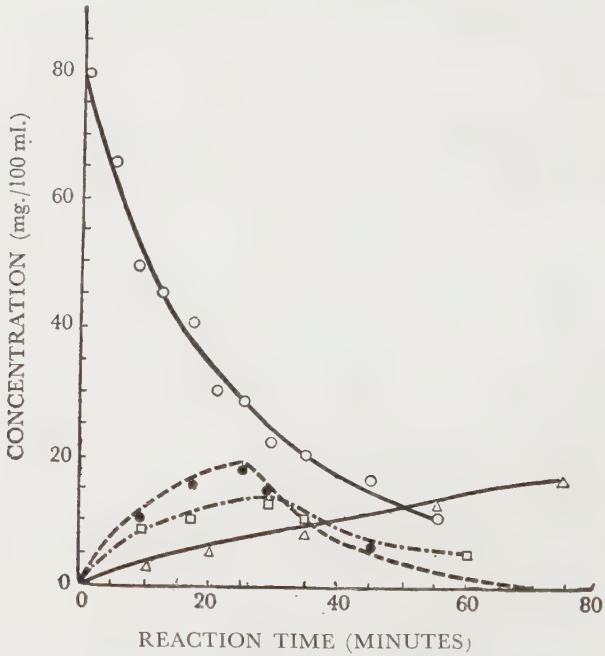


Fig. 7. The action of α-amylase on amylose; changes in the concentrations of the original and the produced amylose and the reducing power.

○ Original amylose; ● the second amylose; □ the third amylose; △ the reducing substance (as maltose)

compare the reaction product of the above experiment with dextrin, dextrin (Kahlbaum), which showed the purple color of the iodine reaction, was purified twice from the mixture of butanol and methanol, and titrated potentiometrically with iodine. The titration curve obtained also showed three breaks just as the reaction product did in the above experiment. An attempt was made in vain to fractionate the three components corresponding to each curve.

The Action of β-Amylase on Amylopectin—Using 25 mg./10 ml. solution of amylopectin as substrate, the following results were obtained: (a) The color of the iodine reaction became red after 60 minutes, and remained without further change; (b) The reaction mixture was titrated potentiometrically with iodine, and the amount of amylopectin at various times was estimated. The reducing power was estimated at the same time. As shown in Fig. 8, when the concentration of amylopectin reached 5 mg./dl. after 60 minutes of the reaction time, the reaction stopped. Maltose produced at this time was 50% of the original amylopectin.

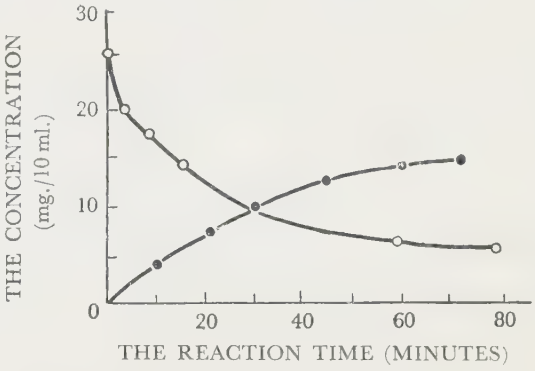


FIG. 8. The action of β-amylase on amylopectin; the changes in the concentration of amylopectin and the reducing power.

○ Amylopectin; ● Reducing substance (as maltose)

The Action of α-Amylase on Amylopectin—By use of 25 mg./10 ml. and 42 mg./10 ml. solutions of amylopectin as substrate, the same conclusion was obtained, from the use of both concentrations. Therefore the findings on the 25 mg./10 ml. solution given below are similar to those on the 42 mg./10 ml. solution. (a) The color of the iodine reaction changed as time went on, from reddish purple to red, to orange after 60 minutes, and finally after 80 minutes the color disappeared; (b) The reaction mixture was titrated potentiometrically with iodine at various times, and the reducing power was also measured. The results are shown in Fig. 9.

The amounts of amylopectin estimated by the potentiometric titration decreased as time went on, and after 80 minutes, it was proved that

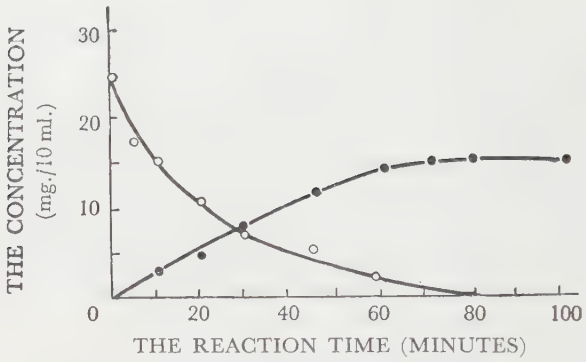


Fig. 9. The action of α-amylase on amylopectin; the changes in the concentration of amylopectin and the reducing power.

○ Amylopectin; ● Reducing substance (as maltose)

the amylopectin had disappeared from the fact that the titration curve now coincided with that of the 0.5 N blank KI solution.

From the relationship between the amount of amylopectin and the reaction time, it could be ascertained that the reaction was of the 1st order in early stage of the reaction.

The Action of "Amylosynthease" on Waxy Rice Strach—(a) The pure solution of waxy rice starch was titrated potentiometrically with iodine. The titration curve showed no indication of amylose present, but it had the same shape as that of amylopectin, as shown by Curve A in Fig. 10. When "amylosynthease" acted upon rice starch solution during

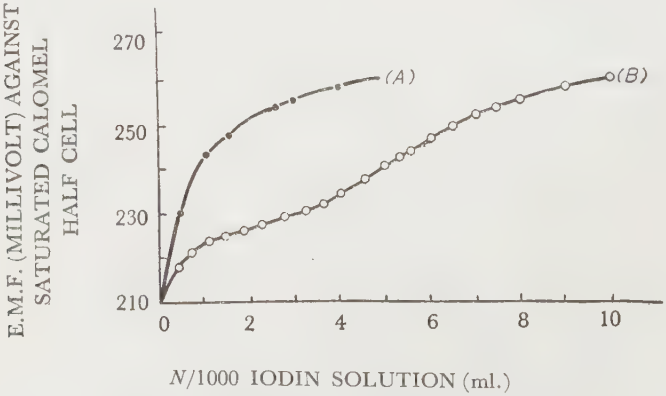


Fig. 10. The action of "amylosynthease" on soluble waxy rice starch; the iodine titration curves

(A) The original waxy rice starch (the control curve); (B) The reaction time; 4 days

48 hours, the potential curves showed some tendency to approach the shape of potential of amylose, indicating that the reaction product had acuqired the property to form a complex compound with iodine. This tendency became more marked after 4–6 days as shown by Curve B in Fig. 10; (b) By the action of the enzyme, precipitates were produced from the reaction mixture. The precipitates were collected, washed thrice with ethanol and dried over sulfric acid. The solution of the precipitates in the concentration of 10 or 20 mg./50 ml. was prepared just like in the case of the amylose solution. This solution was titrated potentiometrically with iodine. Fig. 11, (1) shows the titration curve of 10

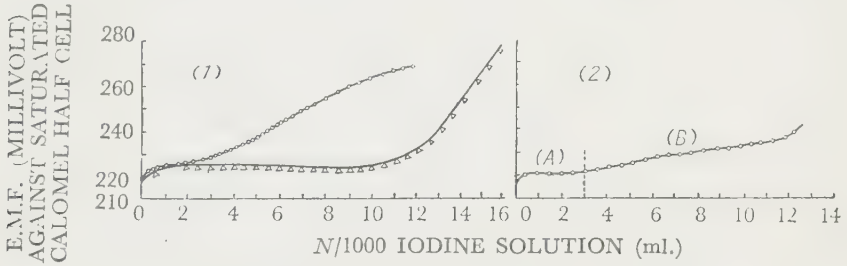


FIG. 11. The iodine titration curve of the reaction product of "amylosynthease."

(1) ○ 10 mg. of reaction product; △ 10 mg. of potato amylose; (2) The mixture of 3 mg. of amylose and 20 mg. of reaction product. (A) The part of the curve indicating the added potato amylose; (B) The part of the curve indicating the reaction product.

mg./50 ml. solution of this substance, compared with that of 10 mg./50 ml. solution of potato amylose. As shown in the figure, the amount of iodine solution required for bringing about the break in the potential curve was far less with the precipitates than with potato amylose. Moreover, in the case of the solution of the precipitates the potential curve did not remain at a definite value of potential, but rose gradually without any definite breaks.

Next, 20 mg. of the precipitates were mixed with 3 mg. of potato amylose and the solution of the mixture was titrated potentiometrically. As shown in Fig. 11 (2), no new break appeared, but the curve rose slowly from the point from which the curve of 3 mg. of pure potato amylose began to rise, showing that the molecules produced were not larger than those of potato amylose; (c) Dextrin of small linear molecules was prepared by making α-amylase act on amylose and precipitating

the reaction product with ethanol. Its iodine color reaction was red, and its titration curve had the same shape as that of amylopectin. The action of "amylosynthease" on this dextrin amylose solution, and on the mixture of the dextrin amylose and waxy rice starch was examined by potentiometric titration. Theye were no curve to suggest that a new linear linkage of amylose was produced from the dextrin amylose.

DISCUSSION

Meyer (*3*) stated that β-amylase separated successively maltose molecules from amylose and that this degradation of maltose proceeded practically to completion. These findings were confirmed by Kerr (*16*), Swanson (*17*) and others. In the present study the action of β-amylose on amylose was observed by potentiometric titration with iodine and it has been found that the potential retains a definite value throughout the course of the enzyme action and that no molecules with different potentials appears. The sum of the degradated amylose and the maltose produced was the same as the amount of the original amylose at any time of the reaction. This result indicates that the enzyme degradates completely one whole molecule of amylose to maltose, before it attaches itself to a new molecule and degradates it. Such an opinion was recently offered by Kerr (*16*) and Swanson (*17*). This experiment confirmed their opinions by establishing the quantitative relationshsp between amylose and maltose. Kerr (*15*) and others stated that the action of β-amylase on amylose was a 1st order reaction, as also confirmed by the present study this conclusion.

As for the action of α-amylose on amylase we have very little knowledge concerning it. Its action is throught to be very complicated, but its main feature lies in the production of shorter chain molecules of dextin accompanied by the liberation of a small amount of reducing sugar in the early stage of the reaction, and later, in the degradation of the peoduced dxtrin to the molecules of reducing sugar. In the present study as the result of the action of α-amylase on amylose, new features of the titration curves were observed, which indicated that the second and the third kinds of amylose were produced. At this stage of the reaction only a small amount of reducing substance was found to be produced. These second and third kinds of amylose have been interpreted to have shorter chain molecules of amylose after the work of Hixon and Foster (*18*). Comparing the present results with the data obtained by them on the relation between viscosity and potentials

of various sorts of amylose, we find that the third amylose obtained above is supposed to have a molecular weight corresponding to that of dextrin amylose prepared by them, which is composed of the number of glucose units of the order of 10^1, whereas the original amylose molecule is composed of the number of glucose units of the order of 10^2. The second amylose is supposed to have a molecular weight between the original amylose and the third amylose. Dextrin (Kaelbaum) seemed to contain the similar sorts of dextrin amylose as the reaction products of this experiment. In Fig. 6 the increasing rate of the potentials near the inflexion points is seen to become smaller as the reaction proceeds, and the sum of the amounts of three sorts of the produced dextrin and maltose to be far less than the amount of the original amylose. These findings indicate that the molecules smaller than those of the third amylose showing no definite potential with iodine are produced as time goes on. When the iodine reaction becomes red, the obtained potential curves are just like the curve of amylopectin and the reaction product in this case consists of still smaller molecules. Probably the molecules of the second and third amylose have been split further by the enzyme. From the results it can be deduced that α-amylase splits the molecules of amylose regularly into molecules of dextrin amylose with a definite molecular weight in the early stage of the reaction, and that afterwards it splits the produced dextrin into still smaller molecules.

Meyer (*3*) stated that β-amylase attached itself to the chain structure of the starch molecule at non-reducing ends and splitted its α-1,6 linkage, producing successively the terminal fragments (maltose), until the reaction stopped at the point of α-1,6 linkage. The residual moleculaes are called "limit-dextrin." In the present study it was observed that the action of β-amylase on amylopectin stopped at a definite time, when there remained residual molecules of "limit-dextrin," which still had some but a less ability to adsorb iodine.

On the other hand there remained no such molecules as "limit dextrin" when α-amylase acted on amylopectin. The result indicates that α-amylase splits amylopectin into smaller molecules which do not from adsorption product with iodine.

As mentioned above, "amylosynthease" was discovered by Nishimura (*5*) and thought to be synthetic enzyme by Minagawa (*6*). The color of the iodine reaction of the substrate was changed from red to blue by this enzyme. Now, from the result of the present experiment it is evident that the enzyme produces the molecules with linear structure such as those of amylose from the branched chain structure of waxy rice

starch molecules, but from the potentiometric titration of the reaction product, it was observed that the product did not consist of a sort of molecule, but of many sorts of smaller molecules, some of which could not form the complex compound with iodine. Comparing the titration curve of potato amylose with that of the mixture of potato amylose and the reaction products, the author concluded that even the largest molecules of the reaction products were smaller than those of potato amylose. When small linear molecules of dextrin were used, the synthesis of new linear molecules of amylose could not be observed at all. Thus the action of this enzyme could not be proved to be synthetic, but all the findings corresponded rather to the opinion of Maruo (*7*) that the enzyme splits the 1,6-linkage of glucoside in starch and produces linear molecules from the branched chain molecules of waxy rice starch.

SUMMARY

1. The action of α-amylase from saliva, and of β-amylase from ungerminated barley on amylose and amylopectin was tested by the potentiometric titration method with iodine. It wsa proved that the amount of amylose and amylopectin in the reaction mixture could be determined by this method. The action of so-called "amylosynthease" from yeast on waxy rice starch was also sested by this method.

2. As the result of the action of β-amylase on amylose, the amount of the original amylose decreased, but the remaining molecules of amylose were the same as those of the original amylose. The sume of the remaining amylose and the maltose produced was the same as the amount of the original amylose. The action was proved to be the 1st order from the observation of both the amounts of the remaining amylose and the produced maltose.

3. By the action of α-amylase on amylose two sorts of dextrin amylose of a definite but smaller molecular weight were obtained at the early stage of the reaction. Later, from these molecules of dextrin amylose of still smaller molecules were produced. The course of these reactions could be determined from the reducing power of the reaction mixture and from the amount of the original amylose remaining and the dextrin amylose produced.

4. As the result of the action of β-amylase on amylopectin, amylopectin was found to be degradated to a certain extent, at times as much as 50%, leaving the molecules of "limit-dextrin" which still had some but a less ability to adsorb iodine.

5. As the result of the action of α-amylase on amylopectin, still smaller molecules were produced with could not adsorb iodine. The course of this reaction was determined from the amounts of amylopectin present and the increase in the reducing powere of the reaction mixture. It was proved that the reaction was of the 1st order at the early stage of the reaction.

6. By the action of so-called "amylosynthease" on waxy rice starch with the branched chain structure, the compounds of linear structure like amylose were produced and their amounts increased with the reaction time. The products consisted of the molecules of different molecular weight and even the largest molecules of the products were smaller than those of potato amylose.

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ON LIPIDS AND TOTAL NITROGEN IN THE CENTRAL NERVOUS TISSUES OF HUMAN FETUS

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The recent advancement of lipid chemistry has promoted the chemical investigations of nervous tissues in many ways. K. Rin and B. Fukuyama reported from our laboratory on lipid aompositions of cerebrum (K. R.), cerebellum, medulla oblongata and spinal cord (B. F.) of dog, particularly their changes in the aourse of growth after the birth. In so far as the author is aware, however, there have been few research reports presented on the changes in the lipid compositions of nervous tissues in human fetues to be brought about with their growth.

Siwertzeff (*1*) estimated the lecithin content of the brain in the human fetus and found the substance which increased with the growth.

Extensive investigations were made on the subject by MacArthur and Doisy (*2*), and later by Cattaeno (*3*), Schuwirth (*4*), and Omura (*5*). Their experiments, however, were conducted on a small number of fetuses and the material examined was resticted to the cerebrum. The present experiments deal with, in a large number of human fetuses, the changes with growth, the distribution of several lipids, and the total nitrogen in cerebrum, cerebellum, medulla oblongata, and spinal cord.

EXPERIMENTAL

Dead human fetuses of different months of pregnancy, taken out for legitimate artificial abortion, were weighed. Their cerebrum, cerebellum, medulla oblongata and spinal cord were separately dissected out as soon as possible after the death.

The homogenates were dried in a high vacuum of about 10^{-2} mmHg, by freezing the homogenates materials with solid CO_2 and acetone.

The dried matter was extracted by Kirck's method (*6*), and the several lipids contained were estimated by the following methods with

aliquot portions of the extract: (a) Total phospholipids and constituents by Erickson's method (*7*); (b) Cerebrosides by Kirck's method (*8*); (c) Cholesteroly by colorimetry.

Total nitrogen was estimated with the dried matter by the micro-Kjeldahl method. In addition, the water and ash contents of cerebrum were determined in the raw homogenate.

Usually each nervous tissue in 5 fetuses of the same months of pregnancy was employed for respective analyses. However, one sample of each tissue was obtained for analyses from 2–5 fetuses when they were in the earlier stages of pregnancy, since the tissue obtainable from one fetus was insufficient in quantity for analyses.

RESULTS

The results of the numerous analyses are shown in Table I–IV, in which, however, only the average values in each month are given.

DISCUSSION

(I). (a) With the growth of fetuses, there is a remarkable increase in the body weight and cerebrum; the ratio of cerebrum to the body weight increases slightly all the time except for a little decrease in the 10th month when.

(b) The water content and the dry substance content on wet basis reaches the maximum and the minimum, respectively, as shown in Table I. According to the general conceptions as abstracted by Peritz (*9*) in 1952, water and protein contents are greater in the grey matter of cerebrum and cerebellem, but smaller in the white matter of cerebrum, medulla oblongata and spinal cord; and in smaller quantities in the grey matter and cerebellum.

(c) The ash content of cerebrum, in general, decreases very slightly in the human fetus, but increases slightly from brith to maturity as the dog grows according to K. Rin (*10*).

(II). (a) *Phospholipids*—With the growth of fetuses, the amount of total phospholipids increases remarkably in cerebrum, medulla oblongata and spinal cord, but very slightly in cerebellum in the latter half period of pregnancy.

(*i*) As reported by Johnson *et al.*, (*11*) cephalin makes up the major parts of phospholipids of the brain in different kinds of animals; it is also the case with the human fetus, their quantity changing in proportion to the amount of total phospholipids.

(*ii*) Lecithin increases gradually in the three tissues excluding cerebellum, in which it rather decreases very slightly, as has been reported by Siwertzeff (*1*).

(*iii*) Schuwirth did not confirm the existence of sphingomyelin in the fetal brain, but the present experiment has ascertained on a reliable evidence that it increases in sphingomyelin slightly in cerebrum, very slightly in spinal cord, gradually in cerebllum with the growth of fetus, but almost unchanged in medulla oblongata,

(b) Except for in cerebellum, cholesterol gradually increase in general, the upward tendency being remarkable after the 8th month, but in cerebellum its increase is very slight. The ratio of total phospholipids to cholesterol declines gradually with the fetal growth in all the four tissues examined, contray to Cattaeno's report (*3*).

(*3*) Cerebroside, the existence of which in the brain tissues was not recognized by MacArthur (*2*), was found to increase remarkably in cerebrum, medulla oblongata and spinal cord, especially after the 9th month. On the contrary, hardly any change is observable in cerebellum during the period of fetal growth. Cerebroside has been considered to be deeply concerned with myelinization of nervous tissues by many chemical and histological investigators; and Omura reported that this process seemed to begin in about the 8th month of pregnancy (5).

(III.) Total nitrogen decreases slightly with the fetal growth in an inverse ratio to the changes of total lipids in three tissues exclusive of cerebellum, where it rather increases in parallel with lipids. The amount of crude potein-N is obtained by subtracting lipids-N from total-N. The crude protein content decreases remarkably in the three tissues, but not in cerebellum, where it is kept almost unchanged in quantity.

As to the relationship between the total lipids and crude protein, the same results may rightly be expected in the human body as in the puppy, as reported by Fukuyama (*12*); that is, the lines indicating the amounts of the two constituents in cerebrum, medulla oblongata and spinal cord cross each other other shortly after birth, but keep running more or less parallel with each other in the case of cerebellum.

SUMMARY

1. Cephalin was found to consitute a principal part of total lipids in the central nervous tissues.

2. The changes in the amounts of lipids and total nitrogen in the

course of fetal growth were very remarkable in nervous tissues, except that lipids increased and total nitrogen decreased considerobly in cerebrum, medulla oblongata and spinal cord during fetal growth.

3. The increase in lipids occurred most conspicuously in cerebrum and less remarkably in medulla oblongata and spinal cord.

4. The lecithin content increased considerably with the fetal growth in medulla oblongata and spinal cord, but in cerebellum it remained almost unchanged; whereas sphingomyelin contet in cerebellum changed quite inversely in quantity.

5. The changes in cholesterol and cerebrosides in amount were most distinct in the last 2–3 months, but for in cerebellum.

6. The ratio of total phospholipids to cholesterol showed a tendency to decline in all the four tissues, but noticeably in cerebrum in the 2nd–4th months; as to that of cephalins to cholesterol, the same changes were also confirmable.

7. The ratio of total phospholipids to cerebroside showed the tendency to decrease in all the four tissues, while that of cerebrosides to sphingomyelin increase to increase except for in cerebellum.

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TABLE I

Lipids, Total Nitrogen, Crude Protein and Ash in Cerebrum, Expresed in mg. per 100 mg. Dry Tissues, together with the Weight of Cerebrum and Bodies on Wet Weight Basis

Month	Body weight	Cerebrum			Ash	Lipids							Lipids -N	Crude protein	Total -N
						Phospholipids				Cholesterol	Cerebroside	Total lipids			
		Weight	Ratio to body weight	Water content		Lecithin	Cephalin	Sphingomyelin	Total						
	g.	*g.*	*g.*	*per cent*											
2	3.0	——	——	90.08	——	3.36	11.30	1.22	15.87	1.05	1.91	18.83	0.34	62.19	10.29
3	——	——	——	90.88	10.05	4.03	13.25	1.40	18.67	1.41	2.20	22.28	0.40	61.94	10.31
4	190.0	21.0	0.01113	90.89	10.31	2.9.	13.87	2.32	19.11	2.23	2.50	23.83	0.41	60.88	10.15
5	480.0	62.0	0.01250	90.97	10.19	3.06	15.90	2.51	21.46	3.04	2.42	26.93	0.46	56.88	9.56
6	539.0	72.3	0.01312	91.09	10.14	3.74	15.73	2.83	22.30	3.15	3.50	28.95	0.49	54.75	9.25
7	1139.0	142.8	0.01273	90.81	9.53	4.11	17.40	3.16	24.67	3.22	3.84	31.73	0.54	52.35	8.92
8	1360.0	193.3	0.01385	90.40	9.42	4.45	19.11	3.21	26.76	3.97	4.26	34.98	0.59	52.25	8.95
9	1833.0	273.0	0.01479	89.61	9.23	5.30	19.08	3.39	27.80	4.47	4.82	37.10	0.62	51.19	8.81
10	2678.0	332.5	0.01238	89.78	9.09	5.94	19.04	3.22	28.20	4.44	6.09	38.74	0.65	51.88	8.95

Table II

Lipids, Total Nitrogen and Crude Protein in the Cerebellum, Expressed in mg. per 100 mg. Dry Tissues

Month	Lipids									
	Phospholipids				Cholesterol	Cerebroside	Total lipids	Lipids –N	Crude protein	Total –N
	Lecithin	Cephalin	Sphingomyelin	Total						
4	4.32	15.87	1.44	21.63	3.23	2.19	27.04	0.45	62.33	10.42
5	4.19	15.61	1.59	21.39	3.05	2.56	27.00	0.45	58.35	9.79
6	3.96	16.31	1.68	21.97	3.35	3.09	28.43	0.48	58.96	9.91
7	3.14	16.86	1.84	21.84	3.53	3.09	28.47	0.48	58.39	9.82
8	3.36	16.26	2.43	22.04	3.74	2.78	28.56	0.48	58.60	9.85
9	3.87	16.23	2.55	22.64	3.66	3.38	29.68	0.50	59.69	10.05
10	3.49	16.16	2.83	22.48	3.56	3.22	29.26	0.49	60.06	10.10

Table III

Lipids, Total Nitrogen and Crude Protein in Medulla Oblongata Expressed in mg. per 100 mg. Dry Tissues

Month	Lipids									
	Phospholipids				Cholesterol	Cerebroside	Total lipids	Lipids –N	Crude protein	Total –N
	Lecithin	Cephalin	Sphingomyelin	Total						
4	1.23	17.60	2.24	21.12	3.92	3.06	28.11	0.46	55.25	9.30
5	2.76	16.51	2.90	22.17	4.07	4.38	30.62	0.51	56.94	9.62
6	3.31	16.55	2.72	22.58	4.13	4.74	31.45	0.53	56.00	9.49
7	3.66	17.40	2.55	23.61	4.60	5.06	33.26	0.55	54.44	9.26
8	4.21	18.00	2.92	25.13	4.53	4.70	34.36	0.57	53.25	9.09
9	4.39	18.84	2.65	25.88	5.73	6.03	39.64	0.61	52.44	9.00
10	5.16	18.88	2.88	26.92	6.05	6.33	39.31	0.64	52.63	9.06

Table IV

Lipids, Total Nitrogen and Crude Protein in Spinal Cord, Expressed in mg. per 100 mg. Dry Tissues

Month	Lipids							Lipids –N	Crude protein	Total –N
	Phospholipids				Cholesterol	Cerebroside	Total lipids			
	Lecithin	Cephalin	Sphingomyelin	Total						
4	2.45	15.55	2.00	20.00	2.88	2.02	24.90	0.42	61.19	10.21
5	3.31	15.45	2.52	21.29	3.08	3.42	27.79	0.47	56.06	9.44
6	3.98	15.91	2.85	22.74	3.14	3.96	29.84	0.50	54.25	9.18
7	4.00	16.93	2.53	23.46	4.64	3.98	32.10	0.52	53.34	9.06
8	4.68	17.02	2.62	24.35	5.49	4.87	34.72	0.56	51.31	8.77
9	4.51	17.59	3.21	25.31	5.51	6.30	37.12	0.61	50.75	8.73
10	4.60	19.12	3.16	26.87	5.74	6.65	39.25	0.64	49.94	8.63

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GLYCOGEN FORMATION AND 2-DESOXY-D-RIBOSE

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2-Desoxy-D-ribose, the carbohydrate of desoxyribonucleic acid, was prepared for the first time from guanine nucleoside by Levne and London (*1*, *2*). We have recently reported on a synthesis of this sugar (*3*) and now we have undertaken the following experiments in order to know whther 2-desoxy-D-ribose has the abillity to form glycogen in livers of mice.

The experiments were made in the spring of 1951. The normal male mice weighing 15–20 g. were used, and fed on the same diet for 7 days. The composition of this diet was as follows: polished rice 60, casein 20, lard 15, McCollum salt (No. 185) 5, dried yeast 5, cod liver oil 2, and water 60. In addition, each animal received 1.5 g. of fresh vegetable every day. Then the animals were fasted for 36 hours in order to reduce the glycogen contents of livers. 1.0 ml. of 2-desoxy-D-ribose solution was then injected subcutaneously and after 2 hours the experimental animals were killed. Livers were removed and the

TABLE I

Glycogen Contents in Livers of Male Mice after 2 Hours Following to the Subcutaneous Injection of Sugar Solutions

The values for glycogen are given in temrs of glucose.

Sugars examined	Number of mice	Concentration of sugars	Average glycogen contents
		per cent	*mg. per cent*
2-Desoxy-D-ribose	13	4.0	862
D-Glucose	15	5.4	2277
D-Xylose	11	4,5	935
D-Abrabinose	11	4.5	745
D-Abrabinal	14	3.5	851
L-Abrabinose	10	4.5	873
Fasted for 36 hours	11	—	772

glycogen contents were determined according to Somogyi's method. (*4*).

Fasted for 36 hours, an average content of 772 mg. % of glycogen in terms of glucose was foudd. As the controls, D-glucose, D-xylose, D-arabinose, D-arabinal, and L-arabinose were examined under the similar conditions. The results obtained are summarized in Table I.

From the above table, the glycogen contents in livers of mice increase definitely following to an injection of D-glucose. There is little evidence that 2-desoxy-D-ribose functions similarly. Neither do other pentoses, except D-ribose (*5*) which has the ability to form glycogen.

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STUDIES ON THE INHIBITORY EFFECT OF SOME -SH REAGENTS UPON THE OXIDATIVE PHOSPHORYLATION IN THE SUCCINATE SYSTEM

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In the previous paper (*1*) the author reported on some of our wartime researches on the mode of action of certain organic halides, whereby we were independently led to the suggestion of the "-SH reaction hypothesis" inasmuch as the combination of these compounds with the -SH groups of the enzyme proteins was inferred to play a significant rôle in their toxicity toward biologial tissues.

The rôle of -SH in the activity of a large number of enzymes were demonstrated through the extensive studies of Barron and Singer (*2*) and other workers (*3*, *4*, *5*, *6*). But it appears that there have been only few researches concerning the effect of -SH reagents upon the oxidative phosphorylation in the Krebs cycle. In this respect it may be pointed out that Ochoa (*7*), in course of his study on the oxidative phosphorylation in the pyruvic-fumaric system, found that the inhibition by iodoacetate ($1\times10^{-3}\,M$) brought about more or less decrease in the P/O ratio, insofar as the P esterification was more slightly inhibited than the oxidation.

The author attempted, therefore, as the first step of the enzymatic re-investigation of the -SH hypothesis, to carry out some experiments concerning the inhibition of the succinic oxidase system by some -SH reagents including heavy metals, with the purpose of elucidating the relative intensity of the inhibition of oxidation and of copuled phosphorylation as well as the reactivating effect of glutathione (GSH) or BAL against the inhibitions in both directions as stated above.

EXPERIMENTAL

Methods—The assay for oxidative phosphorylation was carried out mainly by Potter's method (*8*), some of its individual conditions, being

however, modified according to Ochoa (*9*), Lehninger (*10*), Green (*11*), and Hunter (*12*).

The components of the reaction mixture were as described in Table I.

TABLE I

Inhibition of Oxydative Phosphorylation in the Succinate System by Some Alkylating or Oxidizing Agents

The reaction mixture consists usually of; 30 mg. of creatine in 1.2 ml. H_2O; 0.2 ml. of inhibitor solution with 0.1 ml. of H_2O or activator solution; 0.5 ml. of a mixture of 0.33 *M* KCl, 0.02 *M* phosphate buffer (pH 7.5) and 0.09 *M* NaF; 0.2 ml. of 0.018 *M* ATP (mixed with ADP); 0.2 ml. of a mixture of 0.06 *M* $MgCl_2$ and 0.09 *M* Na succinate (pH 7.5); 0.6 ml. of 14% rat kidney homogenate (total vol., 3.0 ml.)

No.	Inhibitor	Concentration (HIC)	O_2 uptake in 20 minutes	△ Inorganic P	Inhibition of △ inorganic P	Inhibition ratio
	——	*M* 0	*μl.* 143	γ −72	γ 0	(0.42)
1	Alloxan	3×10^{-3}	60	+58	130	1.36
2	Cl-Vinyl Me Ketone	3×10^{-3}	67	+48	115	1.21
3	N Mustard (MBA)	9×10^{-3}	65	+ 4	84	0.78
4	Cl-picrin	2.5×10^{-4}	70	+19	99	0.98
5	Monoiodo-acetate	9×10^{-3}	66	+29	109	1.03
	——	0	150	−80	0	(0.43)

△ Inorganic P: Change of inorganic phosphours after the incubation period; Inhibition of △ inorganic P; Experimental value of △ inorganic P minus control value; (P/O) Inhibition ratio:

$$\frac{\text{Inhibition of } \triangle \text{ inorganic P } (\mu M)}{\text{Inhibition of } O_2 \text{ uptake } (\mu \text{ Atom})}$$

The P/O values of the controls are for convenience presented together in parentheses.

The O_2 uptake was measured in a conventional Waburg apparatus at 30° after 5 minutes of the equilibration period. The determination of "true" inorganic phosphorus was made by the method

of Fiske and Subbarow (*13*). For the purpose of the inhibition studies, the author employed some alkylating (or oxidizing) agents such as methyl-bis (β-chloroethyl) amine (MBA), β-chlorovinyl methyl ketone (VK), monoiodoacetic acid (IAc), chloropicrin (Cp) together with alloxan; in addition some mercaptide-forming agents including heavy metals such as phenyl mercuric acetate (PMA), $Hg^{++}Cl_2$, $Cu^{++}SO_4$, $Cd^{++}SO_4$, and $Zn^{++}SO_4$ for comparison.

RESULTS

I. *Inhibition Studies*:

Alkylating Agents—The experimental data of the inhibitory effect of the alkylating agents described above upon the oxidative phosphorylation in the succinate system are presented in Table I. The final concentrations of the inhibitors were experimentally so selected that the inhibition of O_2 uptake amounted to about one half (50–60%) of the control value (half inhibition concentration of succinic oxidase = HIC).

As shown in Table I, the inhibition of P esterification was found to be more or less remarkable.

In order to elucidate substantially the quantiative relationship between the inhibition of oxidation and of phosphorylation, the author has proposed the "inhibition ratio" (or "P/O inhibition ratio"; see Table I), by which the relative intensity of the inhibition of P esterification against that of O_2 uptake may be given in a more direct manner rather than by the conventional P/O ratio.

The above described inhibition ratio corresponding to the respective HIC was revealed in most cases to be equal to 1–1.2. This would, of course, mean a marked elevation of the ratio from the lower original P/O values (0.42 and 0.43 in both cases).

Heavy Metals—The comparison of the data presented in Table II with the fore-going table reveals on one side that, at common HIC at least, tee qualitative aspect of the status of the inhibition appears to be similar; on the other side, however, it must be emphasized that, in quantitative aspect, the inhibition in both directions is obviously higher in most cases of heavy metals, except phenyl mercuric acetate, than in the alkylating agents already described, and, therefore, the inhibition ratios amount to near 2 or so in the present case.

TABLE II

Inhibition of Oxidative Phosohorylation in the Succinate System by Some Heavy Metals (Intermediate Concentration)

The experimental conditions are similar to those described in Table I.

No.	Inhibitor	Concentration (HIC)	O_2 uptake in 20 minutes	△ Inorganic P	Inhibition of △ inorganic P	Inhibition ratio
	——	M 0	$\mu l.$ 146	γ −166	γ 0	(0.91)
1	PMA	$\leqq 2\times10^{-4}$	59	−28	138	1.28
2	Hg^{++}	5.6×10^{-5}	83	+4	170	2.16
3	Cu^{++}	6.8×10^{-5}	58	+28	194	1.76
4	Cd^{++}	2.5×10^{-5}	53	+35	201	1.72
5	Zn^{++}	0 6×10^{-5}	134 54	−81 +93	0 174	(0.48) 1.74

Further inhibition studies were carried out in order to find out such metal concentrations at which the inhibition of succinic oxidase abolished for the most part, while the inhibition of esterification survived

TABLE III

Inhibition of Oxidative Phosphorylation in the Succinate System by Some Heavy Metals (Lower Concentration)

The experimental conditions are fimilar to those described in Table I.

No.	Inhibitor	Concentration	O_2 uptake in 20 minutes	△ Inorganic P	Inhibition of △ inorganic P	Inhibition ratio
	——	M 0	$\mu l.$ 177	γ −125	γ 0	(0.57)
1	Hg^{++}	1.2×10^{-5} 2.5×10^{-5}	168 158	−114 −52	11 73	(0.96?) 2.8
2	Cu^{++}	1.5×10^{-5} 3.4×10^{-5}	171 134	−102 −20	23 105	3.0? 1.9
3	Cd^{++}	1.2×10^{-6} 6×10^{-6}	174 167	−127 −90	−2 35	? 2.8
4	Zn^{++}	0 9×10^{-6}	130 120	−75 −44	0 31	(0.46) 2.5?

mor or less markedly. In agreement with such expectation, at concentrations ranging from $2-3\times10^{-5}\,M$ (Hg^{++} and Cu^{++}) to lower than $1\times10^{-5}\,M$ (Zn^{++} and Cd^{++}), the inhibition of oxidation falls almost within 10–20%, while the inhibition of P esterification amount to 30–100 γ, as a result of which the inhibition ratios attain such high level as 2 or 3 (Table III).

II. *The Activating Effect of GSH against the Inhibition of Oxidative Phosphorylation by Heavy Metals*:

One of our essential objects in the present studies was to elucidate in what manner this well known natural -SH reactivator of the succinic oxidase would reverse the inhibition of P esterification caused by the heavy metal ions, the results of which may be illustrated in Table IV.

TABLE IV

Reactivating Effect of GSH upon the Inhibition of Oxidative Phosphorylation in the Succinate System by Heavy Metals

The experimental conditions are similar to those described in Table I, except that GSH is added 12 minutes later.

No.	Inhibitor & reactivator	Concentration of inhibitor	Concentration of GSH	O_2 uptake in 15 17 minutes	Reactivation of inhibition of O_2 uptake	Inhibition of inorganic P	Reactivation of inhibition of inorganic P	Inhibition ratio
		M	M	$\mu l.$	%	γ	γ(%)	
	— -	0	0	110		0		(0.62)
1	Hg^{++}	1.4×10^{-4}	0	39		155		1.20
	Hg^{++}+GSH	//	4×10^{-3}	74	49	124	31 (20)	2.85
	——	0	0	110		0		(0.62)
2	Cu^{++}	1.4×10^{-4}	0	21		133		1.26
	Cu^{++}+GSH	//	4×10^{-3}	78	64	144	−11 (−8)	3.6
	——	0	0	134		0		(0.48)
3	Cd^{++}	7×10^{-5}	0	24		192		1.39
	Cd^{++}+GSH	//	2×10^{-3}	52	26	140	52 (27)	1.36
	——	0	0	130		0		(0.46)
4	Zn^{++}	1×10^{-4}	0	39		165		1.44
	Zn^{++}+GSH	//	5×10^{-3}	51	13	183	−18 (−11)	1.86

The reactivation of the inhibition of P esterification was found to take place substantially weaker than that of oxidation and particularly so in the two extreme cases (Cu^{++} and Zn^{++}). The inhibition ratio remained, therefore, unchanged solely in Cd^{++}, while, in other cases, especially Hg^{++} and Cu^{++}, two or three fold elevation was observed.

As the reactivation of both inhibitions, especially in P esterification was found to be considerably incomplete, the author attempted the protection studies with GSH, whereby incidentally a further restoration of irreversible inhibition of -SH enzyme could be observed.

TABLE V

Protective Effect of GSH against the Inhibition of Oxidative Phosphorylation in the Succinate System by Heavy Metals

The experimental conditions are similar to those described in Table I, except that GSH is added before the enzyme is incubated with the inhibitor. The blank O_2 uptake due to GSH alone, as well as its effect upon the oxidative phosphorylation was practically negligible.

No.	Inhibitor and protector	Concentration of inhibitor	Concentration of GSH	O_2 uptake in 16–20 minutes	Protection of inhibition of O_2 uptake	Inhibition of △ inorganic P	Protection of inhibition of △ inorganic P	Inhibition ratio
		M	*M*	*μl.*	%	*γ*	*γ*(%)	
1	——	0	0	127		0		(0.57)
	Hg^{++}	1.4×10^{-3}	0	29		188		1.26
	Hg^{++}+GSH	//	4×10^{-4}	91	63	49	139 (74)	1.08
2	——	0	0	127		0		(0.54)
	Cu^{++}	1.4×10^{-3}	0	42		133		1.26
	Cu^{++}+GSH	//	4×10^{-4}	102	71	98	35 (27)	3.1
3	——	0	0	143		0		(0.40)
	Cd^{++}	7×10^{-5}	0	38		156		1.20
	Cd^{++}+GSH	//	2×10^{-3}	109	69	33	123 (79)	0.78
4	——	0	0	134		0		(0.49)
	Zn^{++}	6×10^{-5}	0	54		174		1.75
	Zn^{++}+GSH	//	2×10^{-3}	78	30	98	76 (44)	1.40
	——	0	0	143	△ inorganic P	−72		
	// +GSH	0	2×10^{-3}	140		−78		

As shown in Table V, the observed protective effect of GSH on the inhibition of oxidative phosphorylation by heavy metals proved to be, in agreement with our expectation, in each case more marked than in case of the reactivation described above. However, the protection of the inhibition of P esterification was found to be more or less higher than that of oxidation, the sole exception being Cu^{++}, in which the protection remained ill-proportionally low. In view of the evidence stated above, it would be quite natural that the inhibition ratios, except with Cu^{++}, decrease more or less, especially in case of Cd^{++}.

III. *The Reactivating Effect of BAL*:

The typical results obtained by using BAL instead of GSH are presented in Table VI.

TABLE VI

Reactivating Effect of BAL against the Inhibition of Oxidative Phosphorylation in the Succinate System by Heavy Metals

The experimental conditions are similar to those described in Table I, except that BAL is added 12 minutes later. The blank O_2 uptake due to BAL alone, as well as its effect upon the oxidative phosphorylation was almost negligible.

No.	Inhibitor and reactivator	Concentration of inhibitor	Concentration of BAL	O_2 uptake in 15 minutes	Reactivation of inhibition of O_2 uptake	Inhibition of △ inorganic P	Reactivation of inhibition of △ inorganic P	Inhibition ratio
	——	*M* 0	*M* 0	*μl.* 120	%	*γ* 0	*γ*(%)	(0.69)
1	Hg^{++}	1.4×10^{-4}	0	24		166		1.38
	Hg^{++}+BAL	//	5.3×10^{-4}	105	84	131	35 (21)	7.0
2	Cu^{++}	1.4×10^{-4}	0	31	67	160		1.44
	Cu^{++}+BAL	//	5.3×10^{-4}	90		145	15 (9)	3.8
3	Cd^{++}	7×10^{-5}	0	25	82	161		1.34
	Cd^{++}+BAL	//	2.7×10^{-4}	103		57	104 (64)	2.64
4	Zn^{++}	1.4×10^{-4}	0	27	60	185		1.60
	Zn^{++}+BAL	//	5.3×10^{-4}	83		148	37 (20)	3.2
	——	0	0	153	△inorganic P		−105	
	// +BAL	0	2×10^{-4}	149			−96	

The reactivating effect of BAL upon the inhibition of oxidation was decidedly greater while the reactivation of the inhibition of P esterification was also brought about by BAL more effectively than by GSH. But the intensity of the latter was consistently far low in comparison with that of the former, as the sequence of with the inhibition ratios in the presence of BAL showed in most cases far conspicuous rise in comparison with the value found in the GSH reactivation.

DISCUSSION

On summarizing the data of the inhibition of succinic oxidase together with coupled P esterification by the -SH reagents (including

heavy metals), it has been revealed an approximate evidence concerning the relative iatensity of both inhibitions. For this purpose, the interrelationship between the HIC of individual inhibitors and the corresponding values of the inhibition ratio are illustrated graphically in Fig. 1. It is evident namely that on one hand the inhibition of P

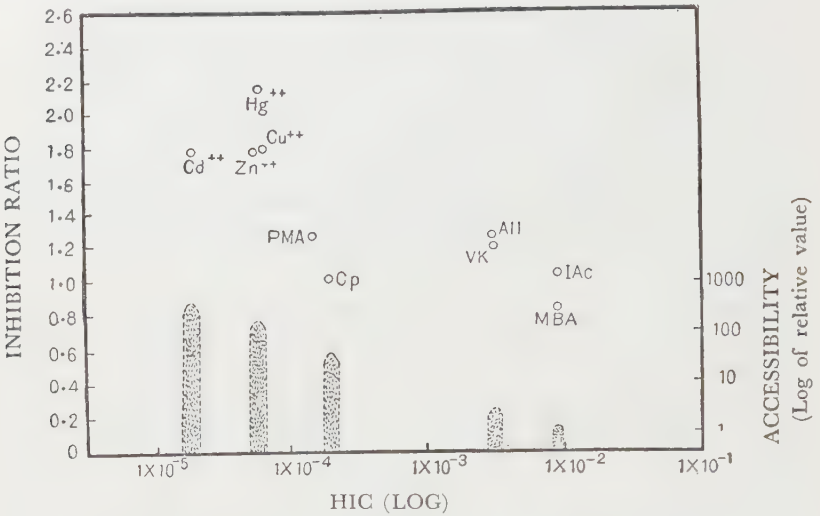

Fig. 1. The interrelation between the half inhibition concentrations of succinic oxidase by several inhibitors and the correspoinding sluggish inhibition ratios, with qualitative representation of accessibility to the -SH groups

IAc: Monoiodoacetate; MBA: Methyl-bis (β-chlorethyl) amine; All: Alloxan; VK; β-Chlorovinyl methyl ketone; Cp: Chloropicrin; PMA: Phenyl mercuric acetate.

esterification in all cases is, more or less, merkedly greater than the inhibition of oxidation, so that each inhibition ratio presented (almost$>$1.0) is far superior to the original P/O ratio value; on the other hand, however, there exists, consistently a significant difference between the inhibition ratios of alkylating agents and of heavy metals. In addition, it would be of some theoretical interest to observe the survival of the dephosphorylating tendency, especially in Cd^{++} and Zn^{++} at concentrations of less than 1×10^{-5} M, similar—perhaps accidentally—to the concentration of decoupling by 2,4-dinitrophenol (DNP) (*11*).

As regards the inhibition of succinic oxidase, β-chlorovinyl methyl ketone, among the alkylating agents studied, which, according to our previous findings (*16*), combined with -SH compounds like cysteine or BAL additionally, inhibited succinic oxidase more strongly than iodoacetate, while the reverse relationship was observed in the endogenous respiration.

Returning to Fig. 1 again, the fact that the HIC of various inhibitors are very widely distributed, might be accounted for on the basis of the geneally accepted hypothesis of -SH groups of various "accessibility," since its qualitative representation is shown graphically. In accordance with the findings of Polis and Meyerhof (*17*), alkylating agents with a weak inhibition such as idoacetate, MBA and VK, are assumed to attack the more superficially located -SH groups which would have nothing to do with the enzyme activity, while the heavy metals such as Cd^{++}, Zn^{++}, Hg^{++} and Cu^{++} with far smaller molecular size, would attack also the less accessible, but probably essential groups which would have decidedly fetal influence upon the enzyme activity.

It must be pointed out that it has been rather unexpected to observe that the activating influences (reactivating as well as protective) of the natural reactivator (GSH) upon four kinds of heavy metals studied show such a considerable degree of deviation that more or less marked individuality can be acknolwedged in the behavior of each metal toward the enzyme system concerned.

SUMMARY

1. As the first step of the enzymatic re-investightion of the "-SH Hypothesis" suggested by us with regard to the action of poisonous gases during World War II, the author undertook some experimental studies on the inhibition of oxidative phosphorylation (modified Potter's method) in the succinate system by some -SH reagents including heavy metals, with the purpose of clarifying the relative intensity of the inhibition of oxidation and coupled P esterification, as well as the reactivating effect of GSH or BAL against both inhibitions.

2. In general aspect, the P esterification was found to be more or less markedly inhibited than oxidation, as the observed elevation of the "(P/O) inhibition ratio" shows (Tables I—III).

3. The comparison of the inhibitory data at HIC *i.e.*, the concentration of the -SH reagents bringing about half inhibition of oxidation (Table I and II) revealed that the inhibition ratios were significantly

higher in heavy metals than in alkylating agents (Fig. 1).

4. The reactivating effect of GSH upon the inhibitions by heavy metals was considerably unsatisfactory, in comparison with its protective effect (Tables IV and V).

5. The reactivation by BAL seems to be somewhat peculiar, since the recovery of oxidation in particular was substantially greater than by GSH (Table VI).

The author is indebted to Prof. Dr. K. Kodama, Tokyo University for his kind suggestions and to Mrs. M. Yamada and H. Kimura for their technical assistance.

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The Journal of Biochemistry, Vol. 39, No. 2,

THE CHEMISTRY OF THE LIPIDS OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES
II. ON THE STRUCTURE OF HEMATAMINIC ACID

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(Received for publication, October 25, 1951)

In has been shown in the preceding paper (*1*), that an apparently new kind of glycolipid, to which the name hematoside was assigned, was isolated from equine blood stroma and a nitrogen-containing polyhydroxy acid was also obtained as a cleavage product of this lipid.

The properties of this acid bear a close resemblance to those of neuraminic acid, a degradation product of ganglioside, which Klenk (*2*) had formerly found in the brain from a case of infantile amaurotic idiocy, bovine brain or spleen, but we designated the substance from hematoside 'hemataminic acid' in order to avoid the confusion of terms.

The molecular composition of hemataminic acid proved to be $C_{10}H_{19}NO_8$ and because of the reducing power after hydrolysis with hydrochloric acid, it was assumed that the substance had acquired a methoxyl grouping as a result of methanolysis during the course of isolation; original non-methoxyl compound, $C_9H_{17}NO_8$, being designated 'prehemataminic acid'.

After the first report went to press, we were able to read detailed reprints concerning ganglioside by courtesy of Prof. Klenk (*3*, *4*). Klenk preferred, in his paper, $C_{11}H_{21}NO_9$ to $C_{10}H_{19}NO_8$, both of which had previously been proposed for neuraminic acid (*2*), and he also noticed the presence of a methoxyl group in it, again designating the non-methoxyl acid, $C_{10}H_{19}NO_9$, as neuraminic acid.

Therefore, the term 'neuraminic acid' is employed by Klenk for two separate substances, but we assume that our prehemataminic acid is identical with the non-methoxyl neuraminic acid and that it is a 9-carbon rather than a 10-carbon compound.

Recently, hexosamine was obtained by Blix (*5*) and identified as chondrosamine on the ground of elementary analysis, optical rotation, and x-ray diffraction pattern.

According to a private communication from Dr. Klenk, which we received some days ago, he found chondrosamine in his preparation of ganglioside and maintained that his neuraminic acid gave, contray to our hemataminic acid, no reducing hydrolysate after cleavage with hydrochloric acid. For this reason, he was doubtful of the identity of these two substances.

In this paper, hemataminic acid is further analyzed and its probable structure is proposed.

As already mentioned, hemataminic acid gave positive reactions with Bial's orcinol and Ehrlich's aldehyde reagents. Molisch's reaction was negative. Neutral solution gave positive ninhydrin reaction. It charred when heated at 220°.

From the results of C, H and N determinations together with similarities of color reactions, it has been considered that neuraminic acid and hemataminic acid might be identical. Moreover, the evidence of identity acquires further confirmation on the ground of optical rotation and titration.

Wherease Klenk (*2*) was of the opinion that it was a monoamino-dicarboxylic acid, we consider it does not adequately express its structure.

Our view is supported by the following reasons: (a) The acidity of hemataminic acid is not so strong as ordinary monoamino-dicarboxylic acid; *i.e.*, hemataminic acid, pH 4.3; aspartic acid, pH 3.3; (b) Formol titration did not show the characteristics of monoamino-dicarboxylic acid; (c) The titration curve of hemataminic acid resembled quite closely that of glucosamine hydrochloride and was different from that of aspartic acid; (d) By the manometric α-amino-carboxyl determination with ninhydrin no carbon dioxide was liberated.

When heated with dilute hydrochloric acid, humin-substance separated out, and the solution became possible to reduce alkaline ferricyanide or Fehling's reagent. The solution after hydrolysis did not restore the color to basic fuchsin which had been decolorized with sulfurous acid, which might be regarded as a proof of the presence of a lactol ring. However, the hydrolysate gave no formic acid on periodate oxidation and the filtrate from the insoluble humin-substance contained no nitrogen, thus representing an occurrence of a considerable decomposition in the molecule. One methoxyl group was found to be present. Nitrogen proved to be amino-nitrogen by Van Slyke's volumetric procedure, which must lie adjacent to hydroxyl group as the result of hydroxyamino-nitrogen determination. No acetyl group was found. Three moles of periodate was consumed per one mole of

the substance and one mole of formic acid was liberated by periodate oxidation. It was detected by color reaction that glyoxylic acid was formed through the periodate oxidation, which indicated the presence of α,β-dihydroxy-carboxylic acid. Schiff's base prepared with aromatic aldehyde, such as vanillin or 2-hydroxynaphthaldehyde, was ether-insoluble; the fact which, according to Jolles and Morgan (*6*), favors the presence of hexosamine-like structure rather than α-amino acid structure.

On the basis of these observations, possible structure for hematominic acid is proposed as shown in Fig. 1.

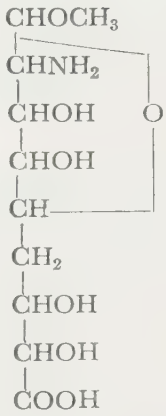


FIG. 1. Hematominic acid

EXPERIMENTAL

Isolation of Hematominic Acid—By approximately the same manner as described in the first report, 190 mg. of crystalline hematominic acid was obtained from 2.0 g. of crude hematoside. The twice recrystallized crystals possessed almost similar properties as those reported by Klenk (*2*) rather than by us in the preceding paper (*1*); namely, whereas the substance in the first report liberated its crystal-water with extreme difficulties, the crystals obtained in the present experiments became anhydrous by drying at 50° in a high vacuum.

Analyses—

Dried at room temperature under moderately reduced pressure in a desicator:

Calcd. for $C_{10}H_{19}NO_8 \cdot 3H_2O$ (335.31): C 35.82, H 7.51

Found: 35.84, 7.20

Dried at 50° under high vacuum over P_2O_5:

Calcd. for $C_{10}H_{19}NO_8$ (281.26):

Diminution of weight 16.12

Found: 16.26, 15.98

Optical Rotation—41.9 mg. of hematominic acid* was dissolved in 1.0 ml. of distilled water, gave in a 1 dm. tube, a reading of −2.27°, hence $[\alpha]_D^{20} = -54.2°$. (Neuraminic acid: $[\alpha]_D^{20} = -54.92°$, (2))

* All the sample used for analysis were dried untill constant weight.

Titration—4.645 mg. of the substance was dissolved in neutral water, with phenolphthalein as the indicator, required 1.59 ml. of 0.01 *N* NaOH.

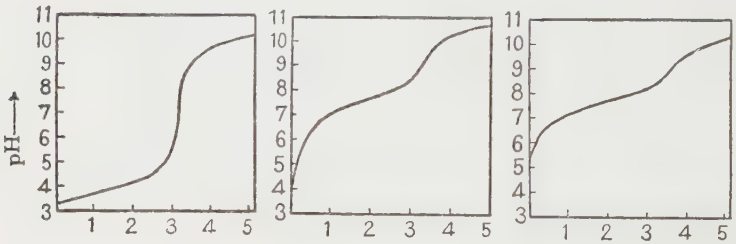
Molecular weight, Found: 291
Calcd. for $C_{10}H_{19}NO_8$: 281
$C_{11}H_{21}NO_9$: 311
(Neuraminic acid: 291 (*2*))

To this neutral solution was added 1 ml. of neutral formalin solution and titration was continued, but the consumption of alkali was only a trace.

5.080 mg. of pure glucosamine hydrochloride repuired 2.36 ml. of alkali (phenolphthalein as the indicator).

Molecular weight, Found: 215.6
Calod. for $C_6H_{14}ClNO_5$: 215.7

Titration Curves—Titration curves were plotted with 8.589 mg. of hemataminic acid, 4.00 mg. (3.01×10^{-5} *M*). of aspartic acid and 6.470 mg. (3.00×10^{-5} *M*.) of glucosamine hydrochloride, using Beckman pH-meter.



Curve I Aspartic acid. Curve II Hematmainic acid. Curve III Glucosamine hydrochloride.

Determination of Amino-Nitrogen—14.640 mg. of the substance liberated 1.45 ml. of N_2 at 26.5°, 759.1 mm-Hg.

Calcd. for $C_{10}H_{19}NO_8$: N 4.98
$C_{11}H_{21}NO_9$: 4.50
Found: NH_2-N 4.88

This result shows that the nitrogen in hemataminic acid is amino-nitrogen, which is in agreement with the result obtained by Klenk (*2*).

Determination of Reducing Power—About 4 mg. of the sample was heated with 0.5 ml. of 5 *N* HCl for 15 minutes. The blackish solution separated by filtration from humin-substance was heated with Fehling's solution on a steam-bath. Red precipitate appeared only after 15 minutes, whereas under the same condition D-galactose reduced the reagent within 5 minutes. Blank procedure gave, of course, no precipitate.

10.030 mg. of the substance was made up to 10 ml. of aqueous solution. For each 1 ml., reducing power was estimated by Hagedorn-Jensen method modified by Wierzuckowski, *et al.* (*7*), after hydrolysis by heating with 5 *N* HCl for various periods. The values were calculated as D-galactose.

After 20 min., 56.0%; 1 hr., 65.1%; 2 hrs., 67.5%.

Distribution of Nitrogen after Acid-Treatment—5.288 mg. of sample was heated with 5 *N* sulfuric acid for 5 hours. Micro-Kjeldahl procedure was worked out with the charcoal-like humin-substance and somewhat blackish filtrate.

Sediment-N 3.95%, Filtrate-N 1.48%

Determination of Carboxyl-Amino Nitrogen—Manometric pocedure of Van Slyke, Dillon, MacFadyen and Hamilton (*8*) was employed.

3.048 mg. of the substance gave 4.9 mm-Hg of PCO_2 at pH 2.5, hence the amount of nitrogen estimated is within experimental error.

Determination of Acetyl Group—7.630 mg. of the substance was saponified with 50 per cent sulfuric acid and distilled in the apparatus of Kuhn-Roth. The distillate required practically no alkali for neutralization.

Determination of Methoxyl Group—Titrimetric procedure of Vieböck and Brecher (*9*) was used. 3.840 mg. of the substance required 7.74 ml. of 0.01 *N* thiosulfate.

Calcd. for $C_{10}H_{19}NO_5$:	OCH_3	11.03
$C_{11}H_{21}NO_9$:		9.97
Found:		10.43

Determination of Hydroxyamino-Nitrogen—The method employed was based on that described by Artom (*10*). 8.040 mg. of the substances was dissolved in 2 ml. of water, 1 ml. of 0.2 *M* periodic acid and 3.5 ml. of borate buffer (pH 9.6) were added and, aster 15 minutes, was placed in a Parnas-Wagner's apparatus. In a receiving flask was measured accurately 5 ml. of 0.01 *N* sulfuric acid. Steam was then passed through

the liquid for 7 minutes. Each 2 ml. of potassium iodide and potassium iodate solution were added and the flask was stoppered. After 5 minutes the liberated iodine was titrated with 0.005 *N* thiosulfate. 5.503 ml. was consumed. The blank required 9.916 ml., hence 4.143 ml. corresponds to ammonia liberated.

Found: N 3.84

Under the similar conditions, L-serine, 11.95 (calcd., 13.33); D-glucosamine hydrochloride, 4.34 (calcd., 6.49); hematoside, 0

Consumption of Periodate during Oxidation—Oxidation was carried out with an aqueous solution of potassium metaperiodate at 7° in the dark. A blank assay was carried out each time under the similar conditions. The consumption of periodate was determined by difference, values being taken on aliquots of the oxidation solution and the blank solution, by titrating with 0.01 *M* sodium arsenite after the addition of sodium bicarbonate and potassium iodide. 2.810 mg. of the substance was made up to 10 ml. of aqueous solution, and analysis was made with each 1 ml. of the solution. The molar consumptions of the oxidant in one experiment were as follows:

15 min., 314; 1 hr., 3.25; 3 hrs., 3.33; 24 hrs., 3.45; 48 hrs., 3.69.

Under the similar conditions, D-mannitol and D-glucosaminic acid consumed the oxidants per 1 mole of substance: 15 min., 4.60, 4.08; 1 hr., 4.73, 4.18; 24 hrs., 4.73 4.22, 48 hrs., 4.73, 4.25; respectively.

Determination of Volatile Acid Produced during Periodate Oxidation—3.440 mg. of the substance was taken up in 2 ml. of water and 20 mg. of potassium metaperiodate was added under ice-cooling. After 10 minutes, the excess periodate was decomposed by addition of five drops of ethylene glycol. The solution was placed in a Parnas-Wagner's apparatus. Steam was passed through the liquid for 30 minutes. The receiving flask was cooled with ice. The collected distillate (about 100 ml.) was titrated with 0.01 *N* NaOH, using methyl red as an in indicator. 1.413 ml. of alkali was required, hence the liberated volatile acid (formic acid) was 1.16 *M*

The excess formic acid over 1 mole was probably due to glyoxylic acid under the influence of periodate. For the purpose to confirm this assumption, volatile acid produced from Rochelle salt during periodate oxidation was determined at intervals. The result is given in Fig. 2.

Accordingly, it became evident that glyoxylic acid formed was converted gradually to formic acid by periodate oxidation.

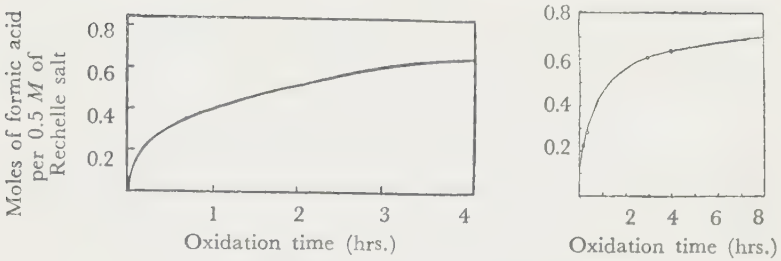


FIG. 2. Periodate oxidation of Rochelle salt

Detection of Glyoxylic Acid after Periodate Oxidation—Qualitative test recommended by Baur (*11*) was applied. About 4 mg. of the substance was dissolved in 0.5 ml. of water, a small amount of periodate, naphthoresorcinol and, after few minutes, one drop of ethylene glycol and concentrated hydrochloric acid were added and heated for 10 minutes on a boiling water-bath. After cooling, the solution was haken with 2 ml. of ether. The ethereal layer became intensely red to violet, indicating the presence of glyoxylic acid. Rochelle salt behaved similarly.

This color reaction is probably not specific to glyoxylic acid, but a substance with both aldehyde and carboxyl group, *e.g.*, uronic acid, would give positive reaction. However, in view of the gradual production of formic acid by periodate oxidation, the formation of glyoxylic acid is rather possible.

DISCUSSION

Since the discovery of hematoside, it was considered to be identical with lignoceryl ganglioside, but the data of C, H and N analyses reported by Klenk (*3*, *4*) and by us (*1*) differed considerably.

In a private communication, Klenk stated that he already found that the glycolipid in the blood stroma resembled ganglioside of spleen and obtained a sugar-rich, neuraminic acid-free lipid from human blood stroma. As its sugar component, he found chondrosamine besides galactose and glucose. As Blix noted (*5*), he found chondrosamine in his preparation of ganglioside; therefore, the molecular formula for ganglioside is no longer adequate.

On the other hand, hematoside gave a fair amount of crystalline hemataminic acid (approximately 10 per cent) and no chondrosamine. But glucose was detected besides galactose by paper chromatography (unpublished data). In view of the value of specific rotation of hemataminic acid, the identity of it with neuraminic acid acquired further

ground, although Klenk claimed that neuraminic acid, contrary to hemataminic acid, gave no reducing hydrolysate with hydrochloric acid. In this report, Fehling's reaction with the hydrolysate of hemataminic acid was shown to be decidedly positive, but the appearance of red precipitate of cuprous oxide was much slower than usual.

Though the configuration of hemataminic acid is not yet fully worked out, the arrangements of hydroxyl and amino groups would probably resemble that of chondrosamine rather than glucosamine.

As shown in the titration curves, the behavior of hemataminic acid towards alkali resembled that of glucosamine hydrochloride, but not that of aspartic acid. Approximately the same was true with ninhydrin reaction in test-tube; namely, aspartic acid gave bluish-violet color with ease, but other two substances did not so without addition of alkali. Hemataminic acid is weakly acidic due to the presence of weak base and strong acid in the molecule.

Provided that the structure of hemataminic acid given in Fig. 1, is right and if splitting might occur with this amino nonuronic acid at C_3, glucuronic acid would be formed and the cleavage product at C_5 be hexosamine.

A theory concerning the biogenesis of hexosamine,* that it might be formed by the condensation of triose and serine rather than from hexose, would support the 9-carbon structure of prehemataminic acid.

The authors wish to express their gratiude to Prof. S. Akiya and Dr. T. Ukita for encouragement. They also thank Miss. R. Ota, Miss. E. Kondō and Mr. B. Kurihara for elementary analyses.

SUMMARY

1. Hemataminic acid, a degradation product of hematoside, was analyzed and an amino nonuronic acid structure was droposed.

2. The identity of hemataminic acid with neuraminic acid was discussed.

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THE STERIC CONFIGURATION OF THE HYDROXYL GROUPS AT C_3 OF THE STEROID MOLECULES*

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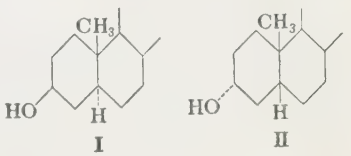
(Received for publication, October 28, 1951)

Based on the so-called Auwers-Skita rule that the catalytic hydrogenation of a carbonly group, according to the neutral and acidic reaction, leads to *trans*- and *cis*-compounds, respectively. Ruzicka *et. al.* (*1*) proposed that the hydroxyl groups at C_3 of the steroid molecules have the following steric configurations (I, II):



I Cholestnol

II *epi*-Coprostanol (bile acids)

The exact opposite structures, however, can be assigned to steroids, if the rule is interpreted in reference to the adjacent methylene group at C_6, despite the hydrogen atom at C_5, as Ruzicka selected for reference. "The reasons," says Sobotka (*2*) in his book criticizing the assumption of the Swiss chemist, "why this and not the opposite structure should be assigned to cholestanol are not cogent," and puts more weight on the considerations of Letter (*3*) than the "dubious" Auwers-Skita rule.

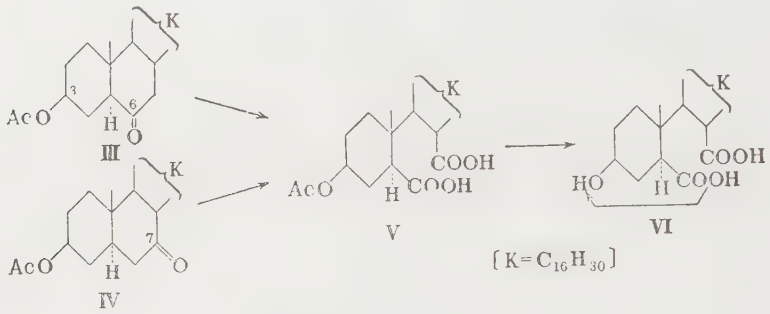
However, the German chemist tried to, determine whether Walden conversion occurred or not, when β-3-hydroxycholestane di-acid was prepared through β-3-chlorocholestane diacid from cholestanolone-6. Applying the rule of Adler-Stein (*4*), that 3-or 4-hydroxycyclohexane carboxylic acid-l is converted into a lactone, only when the relative configuration of the hydroxyl and the carboxyl groups is *cis* to each other, he concluded that the hydroxyl group at C_3 was *cis* (β) to the carboxyl group at C_5 of the β-diacid and that, because the configuration was just the same as that of cholestanol assumed by Ruzicka, no conversion or two consecutive

* The outline of this paper was reported five years ago in the General Meeting of Medicine in China at Nan King (May, 1946) and later in the Meeting of Biochemists in Japan at Kyoto on April 6, 1949.

inversions had occured in these reactions.

To prove if the configuration of the hydroxyl group (C_3) of cholestanol assumed by Ruzicka is correct or not, it is desirable to prepare the β-hydroxy-diacid in an alternate route, which excludes any reactions, suspicious to introduce any conversions of the hydroxyl group.

Fortunately it was successful for us to obtain the very diacid (**VI**) in the following way: 6- and 7-oxocholestanyl acetates (*5*, *6*) (**III**, and **IV**, respectively) were oxidized with fuming nitric acid, which were followed by hydrolysis, that afforded the same hydroxy diacid (**VI**, m.p., 231°*), the ring B being opened between C_6 and C_7, as indicated by the follywing formulas:

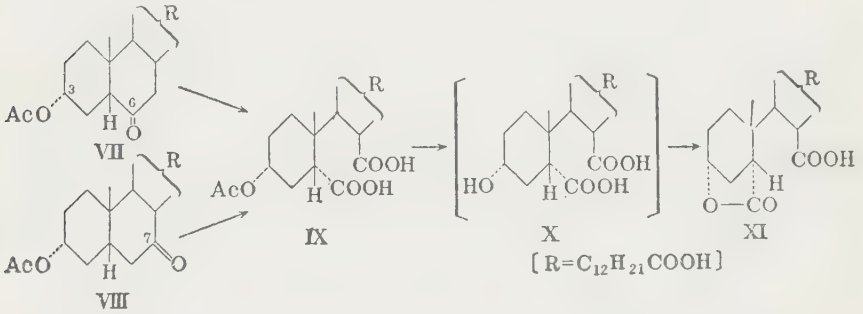


The hydroxy diacid (**VI**), thus obtained, was converted by warming into the same lactone acid (m.p., 211–213°) that Windaus anc Lettré (*3*) prepared. As the hydroxyl group of the hydroxy diacid so obtained underwent no conversion in the course of preparation, it has been thus been thus proved that the hydroxyl group of cholestanol is *trans* to the hydrogen atom at C_5 or *cis* (β) to the methyl group at C_{10}, just as Ruzicka wisely assumed.

The assumption af Ruzicka could also be verified strongly by experiments, with bile acids. Partial oxidation of cheno- and hyodesoxycholic acids afforded 3-hydroxy-6-keto- and 3-hydroxy-7-keto-cholanic acid, respectively (*7*, *8*). These keto bile acids, after protecting the hydroxyl group from oxidation by acetylation (**VII**, **VIII**), were oxidized with fuming nitric acid in the same manner as oxo-cholestanyl acetates. The ring B

* The melting point was found to be a little lower than that of the specimen of Lettre (*3*).

of these acids was thereby opened, both yielding the same tricarboxylic acid acetate (m.p., 258°, **IX**). It is interesting to note that hydrolysis of the acetate affords a lactone diacid (m.p., 229°, **XI**), but not a free hydroxytricarboxylic acid, which is an analogous finding encountered in the preparation of desoxybiliobanic acid (*9*) from chenodesoxycholic acid. So we preferably call the new lactone diacid 'neodesoxybiliobanic acid.' Although, because of such rapid ring closing we could no yet eliminate its hydroxyl group to derive thilobilianic acid (*10*), but here a 3-hydroxy-thilobilianic acid, as clearly demonstrated by the following formulas:



On the basis of the same rule as we have just applied on β-3-hydroxy-cholestane diacid, we can therefore conclude that the hydroxyl group at C_3 of the bile acids is *trans* (α) to the hydrogen atom at C_5 or to the methyl group at C_{10}, just as presented by Ruzicka.

It must be emphasized here that the lactone ring closing of neodesoxy-biliobanic acid is too rapid to catch it in a free hydroxy tricarboxylic acid, just like desoxybiliobanic acid, while β-3-hydroxy-cholestane diacid is obtainable as such. This difference in the easiness of lactone ring closing could be attributed to the different steric configurations of the hydrogen atom at C_5.

EXPERIMENTAL*

(I) *Neodesoxybiliobanic acid*:

Preparation from 3-Hydroxy-6-ketocholanic Acid—3-Acetoxy-6-ketocholanic

* The experiments in the present report were carried out during my stay in Mukden, Manchuria (1945–1948). To my astonishment I have found in Fieser's book (*11*), newly issued, that Kendall (1946) and Shoppee (1948) treated the same problem as we have done arriving at the same conclusions we reached (K.Y.).

acid: 0.7 g. of 3-hydroxy-6-ketocholololanic acid (m.p., 165°) (*7*) were dissolved in 5 ml. of pyridine and the equal volume of freshly distilled acetic anhydride was added. The mixutre was allowed to stand for 24 hours at room temperature and then poured into cold water. At first the reaction product was oily and then became crystalline. It was recrystallized from aqueous alcohol giving platelets, (m.p., 175–176°).

Material, 3.730 mg.: CO_2 9.887 mg., H_2O 3.000 mg.
$C_{26}H_{40}O_8$ Calcd. C 72.17, H 9.33
Found C 72.29, H 9.00

3-Acetoxy-thilobilianic acid: On 0.6 g. of the above keto acetate was poured 1 ml. of concentrated HNO_3 (d=1.48) dropwise under cooling with cold water, until they were dissolved. Then the temperature was elevated slowly up to 40°. After 10 minutes the solution was allowed to stand at room temperature. The oxidation product was deposited in a crystalline paste; it was mixed well with an equal volume of conc. HNO_3 (d=1.38), filtered and washed with nitric acid and then thoroughly with water; giving 0.55 g. of crystals (m.p., 255°). They were rubbed with ether and filtered. Recryztallisation from ethyl acetate gave needles, (m.p., 257–258°).

Titration: 52.0 and 50.0 mg. of the material required 3.24 and 3.10 ml. of *N*/10 NaOH, respectively.
Neutralization equivalent for $C_{26}H_{40}O_8$ (tribasic) Calcd. 160
Found 160.5, 161.3.
Material, 3.830 mg.: CO_2 9.089 mg., H_2O 2.900 mg.
$C_{26}H_{40}O_8$ Calcd. C 64.96, H 8.39
Found C 64.72, H 8.47.

The crystals are soluble in acetone, methanol, ethanol, glacial acetic acid; slightly soluble in ethyl acetate, ether; insoluble in petroleum ether, water. Liebermann's reaction: colorless.

Neodesoxybiliobanic acid: The above tricarboxylic acetate (0.25 g.) in 10 ml. of 2% NaOH-solution was heated. After diluting with water the solution was acidified with dil. HCl and warmed for a while, when crystalline materials were deposited. Recrystallization from aqueous methanol-acetone gave glistening platelets, (m.p., 228–229°).

Titration: 74.3 mg. of the material required 3.60 ml. of *N*/10 NaOH at room temperature and on a water-bath 2.00 ml. more.
Neutralization equivalent for $C_{24}H_{36}O_6 \cdot H_2O$ (dibasic) Calcd. 219
Found 216.4
,, ,, (tribasic) Calcd. 146
Found 132.6

Dimethylester: 50 mg. of the lactone diacid above were neutralized with $N/10$ NaOH (phenolphthalein) and evaporated to dryness. Two drops of dimethyl sulfate were added, warmed for a little while and again two droups of 10% NaOH and water were added, whereby the ester was deposited as crystalline matter. It was filtered and recrystallized from aqueous methanol. Platelets: m.p., 157–158°.

Material, 3.870 mg.: CO_2 9.862 mg., H_2O 3.000 mg.
$C_{26}H_{40}O_6$ Calcd. C 69.59, H 8.99.
Found C 69.50, H 8.67.

The methylester was no longer oxidizable with chromtrioxide, which indicates that there is no remaining free hydroxyl group in its molecule.

Preparation from 3-Hydroxy-7-ketocholanic Acid.—3-Acetoxythilobilianic acid: 3-Acetoxy-7-keto-cholanic acid was preparaed from chenodesoxycholanic acid according to the method of Iwasaki (7). 0.5 g of the keto acid acetate were oxidized with concentrated HNO_2, just as described in the above section. The yield of the tricarboxylic acid was 0.25 g. Recrystallization from ethyl acetate gave needles, melting at 252°. A mixed melting point with a sample of the above gave no depression.

Titration: 75.5 mg. of the material required 4.84 ml. of $N/10$ NaOH.

Neutralization equivalent for $C_{26}H_{40}O_8$ (tribasic) Calcd. 160.
Found 156.

Material, 3.910 mg.: CO_2 9.280 mg., H_2O 3.000 mg.
$C_{26}H_{40}O_8$ Calcd. C 64.96, H 8.39
Found C 64.73, H 8.59.

Neodesoxybiliobanic acid: Saponification of the tricaboxylic acid acetate (0.1 g.) afforded the same lactone diacid, neobiliobanic acid, just, as in the case of 3-acetoxy-6-keto-cholanic acid. Platelets; m.p., 228–229°. A mixed melting point with the lactone diacid prepared from the 6-ketocholanic acid gave no depression.

Titration: 53.8 mg. of the material required 2.62 ml. of $N/10$ NaOH at room temperatore and on the water bath 1.55 ml. more.

Neutralization equivalent for $C_{24}H_{36}O_6 \cdot H_2O$ (dibasic) Calcd. 219
Found 205.3
,, ,, (tribasic) Calcd. 146
Found 129.0

Material, 3.910 mg.: CO_2 9.400 mg., H_2O 3.100 mg.
$C_{24}H_{36}O_6 \cdot H_2O$ Calcd. C 65.71 H8.74
Found C 65.56 H 8.87.

(II) *β-3-Hydroxycholestane diacid*:

Preparation from 6-Oxocholestanol-3—β-3-Acetoxycholestane diacid: 6-Oxo-cholestanol acetate (m.p., 127°) was prepared from cholesteryl acetate according to the method reported by Mauthner and Suida (1903) (*5*). It was subjected to the same HNO_3-oxidation, just as the keto bile acids were.

The resulting product was recrystallized and melted at 220°.

Material, 3.580 mg.: CO_2 9.100 mg., H_2O 3.075 mg.
$C_{29}H_{48}O_6 \cdot \frac{1}{2} H_2O$ Calcd. C 69.32, H 9.61.
Found C 69.41, H 9.85.

β-3-Hydroxycholestane diacid: Hydrolysis of the acetate with NaOH-solution afforded a hydroxy dicarbooxylic acid, melting at 230–231° after recrystallization.

Material, 1.385 mg.: CO_2 3.500 mg., H_2O 1,310 mg.
$C_{27}H_{46}O_5 \cdot H_2O$ Calcd. C 69.17, H 10.33.
Found C 68.92, H 10.598.

Lactone carbonic acid: The dicarboxylic acid was boiled with acetic anhydride and from the acid fraction was obtained a lactone acid, melting after recrystallization at 212–214°. just as Letter (*3*) reported.

Material, 3.600 mg.: CO_2 9.862 mg., H_2O 3.275 mg.
$C_{27}H_{44}O_4$ Calcd. C 74.94, H 10.26.
Found C 74.71, H 10.18.

Preparation from 7-Oxocholestanol-3—7-Oxocholestanol acetate was prepared accordiag to Windaus *et al.* (*6*). The oxidation was carried out just as in the case of 6-oxocholestanol acetate mentioned above.

The analytical data of the resulting hydroxycholestane diacid (m.p., 231–232°) and lactone acid (m.p., 211–213°) are as follows:

β-Hydroxycholestane diacid.
Material, 3.900 mg.: CO_2 9.900 mg., H_2O 3.650 mg.
$C_{27}H_{46}O_5 \cdot H_2O$ Calcd. C 69.17, H 10.33.
Found C 69.23, H 10.37.

Lactone acid.
Material, 3.880 mg.: CO_2 10.642 mg., H_2O 3.563 mg.
$C_{27}H_{44}O_4$ Calcd. C 74.94, H 10.26.
Found C 74.80, H 10.27.

SUMMARY

Applying the rule of Adler-Stein to the experimental data, the authors have proposed to ascertain Ruzick's assumption on the steric

configuration of the hydroxyl groups at C_3 of steroid.

1. Oxidation of 6- and 7-oxocholestanol acetate with fuming nitric acid followed by hydrolysis afforded the same β-hydroxycholestane diacid as prepared through β-3-chlorocholestane diacid.

2. 3-Acetoxy-6- and 7-ketocholanic acids were oxidized by HNO_3-oxidation, yielding the same 3-acetoxy-thilobilianic acid. Hydrolysis of the acetate afforded a new lactone diacid, 'neodesoxybiliobanic acid.'

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STUDIES ON THE INTERACTION BETWEEN PYRIDINE-HEMIN AND HYDROGEN PEROXIDE OR OXYGEN. III. ON THE DECOMPOSITION OF PYRIDINE-HEMICHROME BY HYDROGEN PEROXIDE

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Pyridine-hemin is affected by the action of ascorbic acid and oxygen and a green pigment verdohemochrome is formed through an intermediate compound which is similarly green and designated as 630-compound (*1*). Based on the results of our experimental studies (*1*, *2*) on the mentioned process, it was concluded that the formation of 630-compound was highly dependent upon the amounts of H_2O_2 which was continuously produced in the reaction system of pyridine-hemin, ascorbic acid and oxygen. In the present paper, the results of the direct action of H_2O_2 upon pyridine-hemin especially in the absence of ascorbic acid will be reported. The similar experiment carried out in the presence of ascorbic acid will be postponed for another paper.

EXPERIMENTAL

All the experiments were carried out directly in an absorption cuvette. 30 ml. of pyridine-hemichrome solution were mixed with 0.3 ml. of H_2O_2 solution both varying in concentrations, and the resulting absorption changes were spectrophotometrically measured.

Changes in Absorption Spectra—By the addition of H_2O_2 to the pyridine-hemichrome solution, a profound deformation of its absorption curve was resulted, its characteristic absorption in green region being markedly decreased and in turn a diffuse absorption band being formed in red region with its maximum at about 600 mμ. This maximum at 600 mμ disappeared, if not completely, by reduction with $Na_2S_2O_4$ and there arose in turn two absorption maxima at 557 and 525 mμ. The optical densities, ε_{557} and ε_{525} were found, however, to be much lower than those of the pyridine-hemochrome as would be expected from its initial concentration, and some diffuse absorption band still persisted in red region even after reduction, as mentioned above. The

above >entioned deformation in the absorption curve proceeded roughly in proportion to the concentration of H_2O_2 ndded. These observations indicate, therefore, that the pyridine-hemichrome is decomposed irreversively by H_2O_2, the resulting product being by no means identical either with the 630-compound or with verdohemochrome, as the characteristic absorption maxima of the latter compounds at 630 or 656 mμ have not been found throughout the whole process. Thus, it may be concluded that the 630-compound is not formed from pyridine-hemichrome by the direct action of H_2O_2 alone.

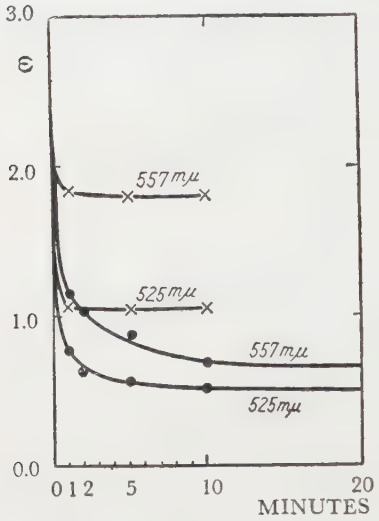

Fig. 1. Decomposition of pyridine-hemichrome through H_2O_2.
$1\times10^{-4}\,M$ pyridine-hemichrome (20% aqueous poridine)
● . . . $5\times10^{-3}\,M$. H_2O_2
× . . . $1.2\times10^{-3}\,M$. H_2O_2

The reaction proceeds in extreme ease, and the time for the reaction to be completed does not exceeds 20 minutes even when a relatively higher concentration of H_2O_2 is applied (Fig. 1).

The Relation between the Rate of Pyridine-Hemichrome Decomposition and the Amount of H_2O_2 Added—3.0 ml. of 1.1 $\times10^{-4}\,M$ solution of hemin in 20% apueous pyridine were mixed with 0.3 ml. of H_2O_2 varying in concentrations. Then, after 1 and 20 minutes each reaction mixture was reduced by adding pulverized $Na_2S_2O_4$, and the optical densities at 557 and 525 mμ were estimated as a measure of the unaffected amount of hemichrome. The observed values of ε_{557} and ε_{525} were plotted against the concentration of H_2O_2 used (Fig. 2). The de composition of the hemichrome, expressed by the diminution of the optical densities at 557 and 525 mμ after the reduction was found to stand proportional to the concentration of H_2O_2 added to the medium. In addition, the curves of the experiment series of both 1 and 20 minutes reaction show a distinct divergence with the increase of H_2O_2. This divergence seems to be related to the reaction rate in each case. The optical density of $1.0\times10^{-4}\,M$ pyridine-hemochrome solution has been determined experimentally as follows: $\varepsilon_{557}=2.8$; $\varepsilon_{525}=1.47$; $\varepsilon_{557}/\varepsilon_{525}=1.90$. Now, if the decrease of ε_{557} is directly proportional to

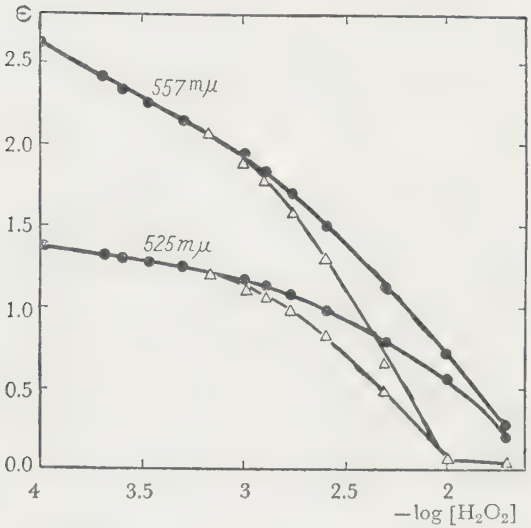

FIG. 2. Decomposition of pyridine-hemichrome and the amounts of H_2O_2 added.
1×10^{-4} *M* pyridine-hemichrome (20% aqueous pyridine)
● . . . Reduced with $Na_2S_2O_4$ after 1 minute of the reaction; △ . . . After 20 minutes of the reaction.

the degree of the decomposition of pyridine-hemichrome, the rate of its decomposition will be calculated by the following equation:

$$\text{Per cent decomposition of pyridine-hemichrome} = 100\times\frac{2.8-\varepsilon_{557}}{2.8}.$$

On the other hand, the rate of its decomposition can be also expressed by the decrease of the ratio, $\varepsilon_{557}/\varepsilon_{525}$. The direct relationship between these two criteria is shown in Fig. 3, calculated from the data given in Fig. 2. As shown in Fig. 3, the ratio, $\varepsilon_{557}/\varepsilon_{525}$ is plotted as a linear function of the per cent decomposition whithin wider range.

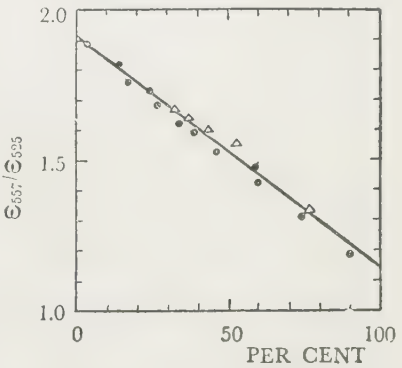

FIG. 3. Per cent decomposition of pyridine-hemichrome and the ratio, $\varepsilon_{557}/\varepsilon_{525}$ in the reaction mixtures after reduction.
● . . . On 1 minute of the reaction; △ . . . On 20 minutes of the reaction.

Thus the reaction product in the present system seems to be a simple substance so far as the linear relationship is maintained. The whole reaction process under the present condition may be expressed, for example, as follows, including the reduction process by $Na_2S_2O_4$:

$$Fe^{+++}\begin{matrix}\diagup\text{Pyr}\\ \diagdown\text{Pyr}\end{matrix} \quad (\longrightarrow x) \longrightarrow \text{A (an end product).}$$

As to x and A, it will be discussed in the later section.

Formation of a Complex Compound of Hemin Derivatives with Hydrogen Perioxide—The complex compound of hemin with H_2O_2 in 100% pyridine was first reported by Haurowitz in 1937 (*3*). On adding H_2O_2 to hemin solution in 100% pyridine, two narrow absorption bands appeared in yellowish green region, which lasted, howeuer, only for a short instant and then the absorption band shifted to form a broad band with its maximum at 557 mμ. These successive displacements of the absorption band were attributed, by Haurowitz, to the possible formation of complex compounds of hemin molecule with two molecules of H_2O_2 in two stages.

In the present studies we have also observed simliar displacements of absorption spectra occurring in the aqueous pyridine, Our findings, however, are not wholly in agreement with that of Haurowitz in 100% pyridine. We have some doubts about his postulation. In the following experiments in different pyridine concentrations and with varying amounts of H_2O_2, the reaction feature will be more precisely studied.

Our findings in 60% pyridine are given in Fig. 4. Just after the addition of H_2O_2 to pyridine-hemin, both dissolved in 60% pyridine solution, the color of the reaction mixture turned at once to brownish green. a diffuse absorption band with its maximum at about 600 mμ being perceived spectroscopically (Fig. 4, I). The proper absorption band of pyridine-hemichrome had almost disappeared at this time. On standing the reaction mixture, the absorption maximum at 600 mμ became more feeble and in turn there appeared gradually two new maxima at 566 and 532 mμ (Fig. 4, II). The displacement began to take place after standing for about 10 minutes, was completed in about 30 minutes and the color of the solution turning again to reddish. The solution remained constant and stable for several hours. The new absorption maxima in green region were not identical with those of pyridine-hemichrome, but the ratio of ε_{566} to ε_{532} was inversed from that of pyridine-hemichrome.

Now, when minimum amount of $Na_2S_2O_4$ was added to the reaction mixture in the state as distinguished in Fig. 4, II (after 60 minutes of the reaction), the color of the solution turned at once from reddish brown to brownish green, showing an absorption curve as given in Fig. 4, III. This is essentially identical with Fig. 4, I in its general feature. On standing, the two maxima at 566 and 532 mμ are gradually restored again. If, however, the solution is vigorously stirred for an instant or a small amount of $Na_2S_2O_4$ is added to it, the restored two maxima will disappear again. When a greater amount of $Na_2S_2O_4$ was added to the mixture in the state of Fig. 4, III, the green color of the solution disappeared and the solution becames reddish again, showing an aborption curve as in Fig. 4, IV. The recoveries of ε_{557} and ε_{525} in the system after the reduction were also dependent on the amount of H_2O_2 used in the intital stage of the reaction.

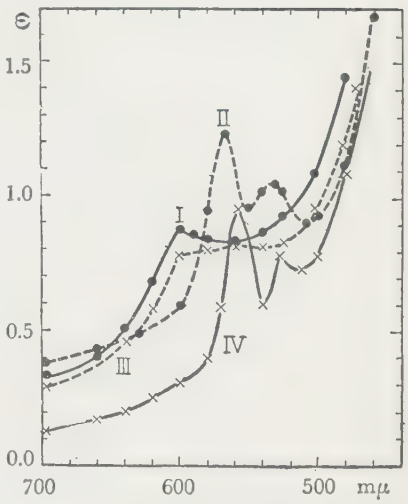

Fig. 4. Spectral changes in the reaction of pyridine-hemin and H_2O_2 in 60% pyridine.
3.0 ml. of $1.5\times10^{-4}M$ pyridine-hemichrome and 0.3 ml. of $1/15\,M$ H_2O_2. II. Immediately after the addition of H_2O_2; II. After 60 minutes; III. The addition of a slight amount of $Na_2S_2O_4$ to I; IV. Further addition of $Na_2S_2O_4$ to III.

The curve of Fig. 4, IV may be regarded, according to our assumption, as a summation of two curves, one of which originates from the pyridine-hemochrome and the other from an unknown compound that is somewhat inert in its spectral activity (corresponding to the compound A, which has been mentioned in the previous section).

Similar results were obtained also in the experiments in 80 or 20% aqueous pyridine, the spectral changes of the latter being somewhat indistinct.

The process in 100% pyridine took place, however, rather in different way from that observed in 60% pyridine. As shown by Fig. 5, I, immediately after the addition of H_2O_2, a sharp absorption band with its maximum at 587 mμ was distinctly observable beside a diffuse

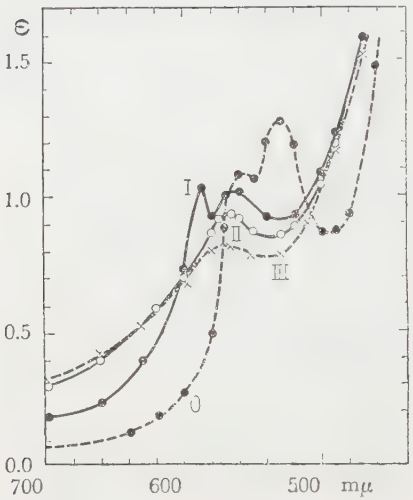

Fig. 5. Spectral changes in the reaction of pyridine-hemichrome and H_2O_2 in 100% pyridine.
3.0 ml. of 1.5×10^{-4} *M* pyridine-hemichrome and 0.3 ml. of 1/20 *M* H_2O_2.
0. Pyridine-hemichrome itself; I. Immediately after the addition of H_2O_2; II. After 10 minutes; III. After 40 minutes.

absorption band with its maximum at 566 mμ. The duration of these two absorption bands was, however, remarkably short, so that already after 30 seconds a clear distinction between these two maxima was no more possible, but there appeared in turn a diffuse and single absorption band in the range of 550–590 mμ. Accordingly, an absorption curve given in Fig. 5, I, may be avaiable only by plotting the optical densities independently measured at each wave length in numerous experiments carried out under the same condition.

In Fig. 5, II, the absorption curve after 5 minutes is given. When the solution was allowed to stand in its III state, its diffuse absorption had gradually decreased. Its absorption curve, after 40 minutes of standing, is shown in Fig. 5, III. Through the whole process of the experiment in 100% pyridine, no appreciable absorption peak was formed in the green region neighboring the wave length of 566 mμ or 532 mμ, at which two distinct maxima were perceived in the process in 60% pyridine. Both reaction processes, in 60% and in 100% pyridine, differed entirely from each other at this point.

The spectral displacement occurring in the process in 100% pyridine has been explained by Haurowitz as follows: the primary absorption bands at 587 and 566 mμ which have persisted within 18–25 seconds after the addition of H_2O_2 should be attributed to a complex compound [Hemin (H_2O_2) (pyridine)]$^+Cl^-$, and the secondary diffus and stable absorption band at 575 mμ in its later stage to a complex compound [Hemin (H_2O_2)]$^+Cl^-$.

No comment was given by Haurowitz on the process in aqueous pyridine. What is meant by the pualitiative difference between the reactions proceeding in the presence and the absence of water in the

reaction medium? The difference may not be essential.

At present we can assume only as follows: pyridine and H_2O_2 campete wite each other to combine with Fe to form a complex compound and their competition may be affected in some way by the presence of water. The experiment with below 90% pyridine will afford some evidence to our assumption. Immediately after the addition of H_2O_2 in 90% pyridine instead of 100% or 80%, we could definitely ascertain the appearance of an absorption band at 587 mμ, which is weak and peristing only for a short instant. On standing, there appear gradually other two absorption maxima at 566 and 532 mμ, similar to those with 80% pyridine. We expected a similar displacement of the absorption to take place, if kept on standing after the addition of a small amount of water to the reaction mixture in 100% pyridine. But so far we have not been able to prove it.

Though our above mentioned assumption is not finally proved, we wil have some discussion to explain the essential feature of the process of the absorption displacement observed in the above experiments. Our assumption is summarized as follows: (a) The compound with its absorption maximum at 600 mμ (its typical absorption is given in Fig. 4, I) is regarded to be some decomposition product of pyridine-hemichrome which is brought about through oxidation by H_2O_2 (possibly corresponding to the *x*-compound denoted in the second section of experimental; (b) The compound given in Fig. 4, II corresponds to the H_2O_2-complex of a substance shown in Fig. 4, I. The substance is, by no means, pyridine-hemochrome, for its absorption disappears by the addition of $Na_2S_2O_4$; (c) Fig. 4, III belongs, in most, to the compound restored from that given by Fig, 4, II through the dissociation of H_2O_2 from its molecule, Fig, 4, III is possibly contaminated with some product of further decomposition; (d) Fig, 4, IV is derived from the mixture of the pyridine-hemochrome formed from pyridine-hemichrome through reduction by $Na_2S_2O_4$ and the decomposition product (presumably the A-compound described in the second section of experimental) of a substance given in Fig, 4, I (presumably the *x*-compound) which is formed through the same reduction process,

The possibility of such a reductive decomposition has once been reported in the previous paper with the fact that a certain unknown compound existing in the verdohemochrome fraction can be decomposed similarly by reduction with strong $Na_2S_2O_4$ (*1*), The above facts are indicating a strong irreversibility of the spectral displacement taking place in the process of the present reaction system,

The figure hterefore, by no means, corresponds to that of a hemin-H_2O_2 complex, the existence of which compound was once postulated by Haurowitz in his experiment in absolute pyridine; (e) The absorptions at 587 and 566 mμ shown in Fig. 5, I belong possibly to the compound $Fe^{+++}<^{Pyr}_{H_2O_2}$ as assumed by Haurowitz. However, we assume that the reaction in pyridine containing water seems to proceed in a way similar to that in the absence of water, but its intermediate figure could be scarcely recognized by our observation, since the reaction in aqueous pyridine seems to pass on almost in an instant; (f) As to Fig. 5, II, any of the explanation will be reserved at present.

On the Substance with its Absorption Maximum at 600 mμ, Denoted as x-Compound—The curves in Fig. 4, I were displaced by Fig. 4, II when kept on standing. This seems to be due to the formation of a H_2O_2-complex of *x*-substance. We will show the following facts for our present assumption. According to Hogness *et al.* (*4*) cytochrome c-peroxidase is brown with its absorption maxima at 500 and 620 mμ. When some H_2O_2 is added to the brown solution, the color turns to red and the absorption maxima at 500 and 620 mμ will be replaced by those at 560 and 530 mμ, where the densities are as follows: $\varepsilon_{560} > \varepsilon_{530}$. The similar observation was reported by Keilin and Mann (*5*) in their experiments with horse-radish peroxidase I. When the peroxidase I with its absorption maxima at 548 and 583 mμ is combined with H_2O_2 molecule, the absorption maxima will be replaced by those at 530.5 and 561 mμ, ($\varepsilon_{561} > \varepsilon_{530\cdot5}$). These absorption displacements followed to the formation of H_2O_2-complex compound have a close resemblance, in their nature, to those observed in our present investigation.

On the other hand, Stern has reported on the existence of ethyl-peroxide complex compound of catalase (*5*). This complex was reinvestigated recently by Chance more precisely (*7*). It seems to be of some interest to point out that, while it is difficult to perceive the formation of the H_2O_2-complex compound of catalase, its C_2H_5OOH-complex is easily observable. The difference may perhaps be based on the fact that H_2O_2 is decomposed much more easily than C_2H_5OOH, both in their complex state. The existence of hemiglobin-H_2O_2-complex was als reported by Haurowitz (*8*). The finding seems also to be connected with the fact that the catalatic activity of hemiglobin is much lower than that of catalase. By analogy with the above considerations it may be postulated that the catalatic activity of pyridine-hemin is very weak in

100%pyridine and so it is very stable against H_2O_2.

For the formation of a complex of α-compound with H_2O_2, a certain excess of H_2O_2 may be required in the medium. And this may be possible only under the assumption that the substance with its absorption maximum at 600 mμ possesses a considerable stability against H_2O_2. As a matter of fact, the figure of the H_2O_2-compound (Fig. 4, II) is recognizable for a considerable time without any change (18 hours). It could sill be distinctly observable on the next morning. In our previous paper we have reported on the great stability of the 630-compound against H_2O_2. In analogy with the fact, we assume that the substance characterized by its absorption maximum at 600 mμ may be similarly resistent against H_2O_2, although nothing is known at present about its chemical structure.

SUMMARY

1. The product formed by the direct action of H_2O_2 on the pyridine-hemichrome solution is not the 630-compound, but likely to be another new degradation product of pyridine-hemin, characterized by its absorption maximum at about 600 mμ.

2. In the course of the reaction process, a H_2O_2-complex of the new decomposition product was observed spectroscopically. On the basis of our observations, some discussion have been made against the [Hemin $(H_2O_2)_2]^+Cl^-$ compound postulated by Haurowitz.

3. The new decomposition product posesses a considerable stability against H_2O_2 and a relatively weak catalatic activity.

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SOME MICROMETHODS FOR ENZYME STUDIES

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In the course of the studies on enzyme chemistry in this laboratory, the author came to devise for some sorts of micro-methods for quantitative determination of biological substances. These methods were proved to serve fairly well for the purpose, so that they are to be published here.

I. Phosphate (By Kishima IMAI)

Colorimetric method of Fiske and Subbarow, which is used widely, is not sensitive enough to measure the initial velocity of phosphatase action or to determine P content in 0.1 ml. of blood. King and Delory (*1*) proposed a new method of estimating inorganic phosphate by the application of Folin-Ciocalteu's reagent for the colorimetry of hydroxy quinoline (oxine), phosphate being precipitated previously as phosphomolybdate by the addition of acidified oxine molybdate solution. With this method, however, a high sensibility can not be expected, because it based on the colorimetric principle due to molybdene blue. We used now, instead of oxine, some organic dyes which are able to be precipitated by phosphomolybdate, but not by molybdate; and they were phenosafrainine and its dimethyl derivative, namely methylene violet. According to our experience methylene violet is better suited for colorimetry by Duboscq's apparatus, because its color is deeper than that of safranine; whereas the latter is preferable for the photometry by Pulfrich's apparatus, since its color fits the measurement using filter S_{53}.

In this dye-molybdate method deproteinization should be done by sulfosalicylic acid instead of trichloroacetic acid, because the latter precipitates the dye. Hydrolysis for the determination of acid soluble P in the deproteinized filtrate was carried out by heating with concentrated hydrochloric acid instead of sulfuric acid in a quartz test tube or a Pyrex tube to avoid contamination of silicic acid from softglass.

Reagents—(a) 5 *N* and 1 *N* HCl. Concentrated hydrochloric acid is diluted with an equal or nine volumes of water; (b) Dye-molybdate

solution. One volume of 5 *N* HCl, one volume of 4.2% ammonium molybdate and three volumes of 0.1% methylene violet (Grübler) or safranine solution are mixed and filtered through a Toyo filter 5c on the next day. Any precipitate appears during storage should be filtered away; (c) Five % apueous sulfosalicylic acid solution; (d) Acetone; (e) Standard phosphate solution. A solution of 0.439% KH_2PO_4 is diluted to 1:1000, 1 ml. of this solution contains 1 γ of phosphorus.

Procedure—For blood or serum analysis 0.1 ml. of the specimen is poured into 10 ml. of water, the pipette thereby being washed out twice with the solution, and 2 ml. of the diluted blood or serum are then mixed with 2 ml. of sulfosalicylic acid solution and filtered through a Toyo filter 5c. In the case of phosphatase assay with dialyzed enzyme solution a very dilute solution of substrate, for example sodium pyrophosphate or β-glycerophosphate, can be used, and 2 ml. of the test solution are to be deproteinized directly without previous dilution for the determination of the liberated P.

Inorganic Phosphorus Estimation—Two ml. of the deproteinized filttrate are mixed with 4 ml. of the dye-molybdate solution and centrifuged after standing for 10 minutes. The colored supernatant is removed away as usually by gentle suction through a glass capillary. The precipitate is washed further twice with each 4 ml. of 1 *N* HCl by centrifugation, dissolved in acetone and filled up with it to the 10 ml.-mark. For running the colorimetry of the violet solution by Duboscq's apparatus the standard solution is prepared as follows: 1 ml. of the dilute phosphate solution is mixed with 1 ml. of sulfosalicylic acid, then with 4 ml. of the dye reagent, and the centrifugation, washing and dissolving the precipitate are carried out in the above mentioned manner. The calculation of P content per ml. of the tested blood or serum is as follows:

$$\text{Inorganic P} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 100\,\gamma/ml.$$

In the case of photometry by Pulfrich's apparatus (the filter S_{53}) the extinction coefficient is 0.510 for the color developed in the above mentioned way out of 1 ml. of *M*/40,000 phosphate solution, 1 ml. of sulfosalicylic acid and 4 ml. of the safranine reagent.

Acid Soluble Phosphorus Estimation—One ml. of the deproteinized serum filtrate is added to 1 ml. of concentrated HCl and heated over a small gas flame for 5 minutes to be concentrated to 0.2 ml., cooled and the remaining small amount of yellow fluid is mixed with 2 ml. of water, boiled again till the volume is reduced nearly to 1 ml. The content is transferred quantitatively into a centrifuge tube and mixed with 4 ml.

of dye-molybdate solution. The subsequent procedure is the same as in the inorganic P estimation.

$$\text{Acid soluble P} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times 200\,\gamma/ml.$$

Results—Each 1 ml. of 1, 1/2, 1/4, and $1/8 \times 10^{-4}\,M$ solution of sodium β-glycerophosphate was analyzed, and 99.5, 97.7, 99.9 and 100.5% of theoretical values were obtained, respectively.

The distribution of P in blood has been measured as follows: 0.1 ml. of blood is diluted with 10 ml. of physiological salt solution. Two ml. of the diluted blood are used for the determinations of inorganic and acid soluble P in whole blood; 5 ml. of the remaining diluted blood are centrifuged, and 2 ml. of the supernatant are then used for serume P determination. The precipitated blood cells are washed twice with physiological salt solution, hemolyzed with water, diluted immediately to the 5 ml,-mark, and 2 ml. of the hemolyzed solution are submitted to P analysis. Estimation is carried out with two samples of human blood.

Distribution of Phosphorus in Blood

	Inorganic P		Acid soluble P	
No. of sample	I	II	I	II
Total blood	3.75 *mg%*	3.63 *mg%*	14.28 *mg%*	14.50 *mg%*
(A) Plasma	2.40	2.38	5.76	4.65
(B) Blood cell	1.38	1.24	8.92	9.60
(A)+(B)	3.78	3.62	14.68	14.25

II. Phenol (By Yasuyuki SHISHIKURA)

In this laboratory *p*-nitrophenol derivatives have been used as substrates for phosphatase (*2–5*), sulfatase (*7*, *8*) and heterosidase (*9–12*). These compounds are in general more easily hydrolyzable than other corresponding derivatives, and moreover the estimation of the liberated *p*-nitrophenol can be carried out by a simple procedure and quantitatively even in extremely dilute solutions. This method is now becoming popular in foreign countries. However, we have encountered often the cases, where phenol should be estimated, for example to compare the hydrolysis of phenol and *p*-nitrophenol phosphates with each other to investigate the hydrolysis of di-phenol pyrophosphate. Hence we have tried a modification of the method of Gilbbs (*13*, *14*). 2,6-Dibromo-4-aminophenol chloride (DBAP) is used. This reagent is mixed with

phenol and adjusted to pH 9.0 by borax buffer, and to the mixture is added an oxidant, potassium ferricyanide. Then the intermediately formed dibromoquinone imide reacts with phenol to produce a clear indophenol solution. The extinction is proportional to the phenol concentration. Colorimetry by Duboscq's apparatus can not, however, be carried out because of the contaminated color of ferricyanide. This disadvontage can be avoided, if potassium periodate is used as the oxidant. Another disadvantage is that the indophenol formation is inhibited more or less in the presence of a large amounts of organic substances, for example protein or amino acids. Organic buffere solution such as veronal or acetate, and a small amount of amino acid are, however, indifferent.

Reagents—(a) DBAP solution. 100 ml. of water are acidified to react neutral agaiust Congo paper with hydrochloric acid and 0.1 g of DBAP is dissolved in it by warming. The solution can be useful for several months; (b) Borax solution. Saturated borax solution (nearly 3%) is adjusted to pH 9 by the addition of conecentrated hydrochloric acid; (c) 10% potassium ferricyanide solution.

Procedure—To 1–3 ml. of the test solution taken in a calibrated 10 ml. tube provided with the glass stopper, 1 ml. of borax is added and, if necessary the solution is adjusted to pH 9 by dropping in dilute NaOH or HCl from a capillary pipette, being tested against thymol blue paper. Two-tenths ml. of ferricyanide and then 0.2 ml. of DBAP solution are added, warmed at 37° in a water bath for 5 minutes and diluted with water to the mark. Within 10 minutes to one hour after the dilution the photometry with filter S_{61} should be run. Extinction (10 mm.) of the blue solutions out of 1 ml. of *M*/2000 phenol is 1.171.

Remarks—This method was intended at first to be applied to the determination of phenol in urine. It was found, however, soon that DBAP reacted also with aniline, anthranilic acid and salicyluric acid. Salicylic acid of high dilution had remained colorless after the addition of DBAP reagent and subsequent oxidation; but developed a clear blue color, when the solution was made alkaline by adding 1 ml. of 2% NaOH. This color could not be extracted with butanol in contrast to those from other substances. On the other hand indamine derived from aniline was a single dye extractible with xylene at pH 9. The extinction coefficients of the colored solutions, obtained from each 1 ml. of *M*/2000 solutions, were as follows: Indamine was extracted with 5 ml. of xylene, whereas other dyes were diluted simply with water to 10 ml., as above mentioned in the case of phenol.

Substance	Phenol	Salicylic acid	Salicyluric acid	Anthranilic acid	Aniline (in xylene)
Extinction coefficient (10 mm.)	1. 172	0. 416	1. 420	0. 432	0. 430

In order to analyze the DBAP positive substances in urine, the previous removal of urinary colloid was necesary and, since the colors derived from phenol, salicyluric acid and anthranilic acid could not be separated from each other—moreover even salicylic acid, if in such a high concentration as *M*/200 showed at pH 9 a slight blue color—a rather complicated procedure for separating various fractions according to their different solubilities in organic solvents had to be carried out prior to the color development with DBAP reagent. This procedure has been reported in detail in Japanese by Shishikura, the description of which being committed here.

Tyrosine, histidine, urice acid, tryptophan, kynurenine, *o*-amino acetophenone and pyridine gave no coloring even after the addition of formation alkali. Hence this method may be convenient to traee the phenol from tyrosine by bacteria.

III. Ammona (By Ichro KATSUMATA)

The method is based on the reaction of Thomas, namely indophenol formation by the reaction of hypochlorite in alkaline solution with the mixture of phenol and ammonia. This reaction has been applied already by van Slyke and Hiller (*15*), Borsook (*16*) and Russel (*17*) to the quantitative estimation of ammonia. According to these authors, however, the hypochlorite solution must prepared from calcium salt, and should be tested for the content of available chlorine and be stored in portions. In spite of those treatments, which are inconvenient in practice, ammonia solution of a definite concentration must be used simultaneously either to prepare the standard for colorimetry by Duboscq's apparatus or to ascertain the expected extinction coefficient of the standard solution produced. We have intended to make the method somewhat simpler.

Reagents—(a) Phenol solution. 5 g. of phenol are dissolved in 95 ml. of water and stored in a brown bottle; (b) Bicarbonate solution. 10 g. of sodium bicarbonate are dissolved in 100 ml. of water without warming and, if saturated, the supernatant may be used; (c) Antiformine-soda solution. Two volumes of antiformine (sodium hypochlorite solution,

Hirazawa) and one volume of 10.6% sodium carbonate solution are mixed and the supernatant is stored in an ice-box; (d) *M*/1000 ammonium sulfate solution for standard.

Procedure—To 1 ml. of ammonia solution of unknown concentration in a calibrated test tube immersed in ice-water, 2 ml. of phenol solution, 1 ml. of bicarbonate solution and 3 ml. of antiformine-soda mixture are added successively, mixed thoroughly, left at room temperature for 20 minutes and filled up with water to the 10 ml.-mark. To get rid of a possible contamination of ammonia in the reagentus, 1 ml. of water, instead of the test solution, is mixed with the reagents in the above mentioned way, served as control after being diluted to 10 ml. In the case of photometry by Pulfrich's apparatus, the scale on the side of the control solution is fixed at zero. Extinction coefficients of the blue solutions are proportional to the ammonium concentration within the rang of *M*/1000-*M*/8000, when each 1 ml. of ammonium sulfate solution is used. Since, however, the absolute value of extinction may vary as a result of alteration of the antiformine reagent, 1 ml. of *M*1000 ammonium sulfate must be colorized simultaneously in the way as mentioned above for the calculation of ammonium concentration in the test solution. E (10 mm.) of the solution out of 1 ml. of *M*/1000 solution is in general, 0.730~0.750.

Remarks—This method can not be applied directly to the test solution, because protein, but Amino acids show also indophenol formation to a more or less extent. Therefore the distillation of ammonia should be carried out previously, and for the micromethod as in this case, the diffusion cell of Conway was very conveninet, as Borsook and Russel has suggested already. We could thus determine urea in 0.1 ml. of blood or serum by the urease method. We verified further by this method, that histidase (*18*, *19*) could liberate 100% ammonia from histidine. On the other hand the possible reversed reaction—the enzymatic histidine formation from urocanic acid and ammonia—was proved never to occur, being judged by the method for histidine estimation of Oyamda (*20*). It did not occur also in the cases of experiments with kidney or liver brei in the presence of ammonia or any other ammonia sources, for example aminophosphate, glycine, alanine, asparagine or glutamic acid.

SUMMARY

Micromethods for quantiative estimation of phosphate, phenol and ammonia have been described. They are applicable to enzyme studies

with dilute substrate solutions or to the measurements of initial velocity of reactions.

1. Inorganic phosphate is measured by colorimetry of methylene vioelet or safranine in the acid-insoluble dye phosphomolybdate, which is obtained by the addition of dye-molybdate to 1 ml. of phosphate solution in acidic solution, washed with hydrochloric acid and dissolved in 10 ml. of acetone. Either photometer or Duboscq's colorimeter may be used. Deproteinization should be done by use of sulfosalicylic acid. Acid soluble P can be determined in the hydrolyzate of the filtrate with hydrochloric acid. The method applicable to the sractional estimations of P in 0.1 ml. of serum.

2. Phenol is determined by carrying out the photometry of indophenol, produced at pH 9 by the addition of 2,6-dibromo-4-aminophenol chloride and potassium ferri cyanide (or periodate). In this case the extinction coefficients are also proportional to the phenol concentrations. The principle for isolating the phenol fraction from other substances is described briefly.

3. Reagents for ammonia estimation by phenolhypochloric procedure are modified to make their preparation easy,

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STUDIES ON CYSTEINASE*

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It has been shown by Tarr (*1*) that cysteine is decomposed by microorganisms, especially by *Proteus vulgaris*, with the prodution of hydrogen sulfide, acetate, formate and ammonia. Desnuell *et al.* (*2*, *3*, *4*) observed an enzyme system in *E. coli*, in which hydrogen sulfide was produced from cysteine. Moreover, Tarr (*5*) has drawn deductions regarding the mechanism of the formation of hydrogen sulfide by *Proteus vulgaris*. Using various sulfur containing amino acids and their derivatives, he concluded that α-amino-β-thiolcarboxylic acids served as the sources for hydrogen sulfide.

Recent investigations in the literature as well as in our laboratory, have revealed that the microbial degradation of tryptophan and tyrosine gives rise to indole and phenol, respectively, through primary fission of the intact side chain, instead of through more probable stepwise intermediates (*6*, *7*, *8*). Further evidence for primary fission of the intact side chain has been reported with reference to methioninase, the enzyme producing methylmercaptan from methionic or its α-hydroxyderivative (9). From these results, it seems that the above mentioned mechanism of the primary fission would be more plausible in the case of hydrogen sulfide formation from cysteine. After possible intermediates of cystine were investigated by using *Proteus vulgaris***, it was confirmed that cystine, cysteine and its α-hydroxy-derivatives could serve as sole sources of hydrogen sulfide. In relation to those bacterial dissimilations, therefore, the same mechanism of the degradation as for tryptophanase, methioninase, β-tyrosinase (related to phenol formation from tyrosine) and cysteinase was suggested.

Umbreit *et al.* (*10*) has more recently worked out the purification

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and the resolution of tryptophanase and has reported its reactivation by pyridoxal-phosphate. It was later shown in our laboratory that pyridoxal and adenosine-triphosphate (ATP) could act as a coenzyme both for β-tyrosinase (*11*) and methioinase (*12*). In considering the probable role of pyridoxal-phosphate as the coenzyme of cysteinase, the adaptive dried cells of *E. coli* grown on crysteine were employed as a source of cysteinase. This was partially purified by several steps including fractional precipitation with ammonium sulfate of cell-free juices, absorption on kaolin and subsequently resolved by treatment with cyanide. The resolved enzyme regained its activity when supplied with both pyridoxal and ATP.

Furthermore, Fromageot *et al.* (*13*, *14*) found a system in dog liver which attacked cystenine to produce hydrogen sulfide, catalyzing the following reaction:

$$3\ \text{Cysteine} \longrightarrow H_2S + \text{Alanine} + \text{Cystine}$$

Smythe (*15*) reported that, by using the enzyme in rat liver, the conditions favoring the removal of hydrogen sulfide in accord with the following equation was obtained.

$$\text{Cysteine} \longrightarrow H_2S + NH_3 + \text{Pyruvate}$$

This paper is also concerned with the elucidation of the related mechanism of the reaction involved on the basis of the properties of the enzyme, the demonstration of alanine, and the determination of pyruvate and ammonia as metabolites.

EXPERIMENTAL

1. H_2S Formation from Cysteine (Cystine) and its Derivatives:

Materials and Methods—The strain of *Proteus vulgaris* X_{19} was a stock culture of the departmental collection of the Osaka University. The organisms were grown on agar-plates for 24 hours, harvested, washed with distilled water by centrifugation and suspended in *M*/15 phosphate buffer at pH 7.6: (20 mg. of dry weight organisms per ml.).

The hydrogen sulfide evolved was tested by use of the color-change of filter paper previously wetted with 10 per cent lead acetate attached to the coton-stoper of the flask. It was also determined by measuring the level of color-development according to the methyleneblue method (*16*), which was carried out in a glass stoppered bottle exuipped with a system for aeration and in which hydrogen sulfide was absorbed in 0.6 per cent zinc acetate.

The sulfur containing compounds in this study were L-cystine (commercial), L-cysteine-HCl (by reducing L-cystine with tin and HCl), L-α-dihydroxy-β-dithiodipropionic acid (*17*), β-dithio- dipropionic acid (*18*), dithiodiglycolic acid (*19*), L-cysteic acid (*18*), L-N-benzoylcylcystine (*20*), taurin (commercial), and Na thioglycolate (commercial).

The compounds mentioned above were dissolved in distilled water in a concentrations of 1 mg. per ml., and sterilized throgh a porcelain filter (Chamberland L_3). In case of cystine, however, the same concentration was mad in *N*/100 NaOH, filtered through the Chamberland L_3, and adjusted to nearly pH 7.0 with 10 per cent HCl.

Results—In this study, the formation of hydrogen sulfide by microbial degradation was made using 2 ml. of the above compounds, respectively (as listed in Table I), 2 ml. of *M*/15 phosphate buffer (pH 7.6), and 2 ml. of cell-suspension.

Hydrogen sulfide was produced only from L-cysteine, L-cystine, and L-α-hydroxycarboxylic acid, but not from N-benzoyl-L-cystine, β-dithiodipropionic acid, dithiodiglycolic acid, Na thioglycolate, L-cysteic acid or taurin (Table I).

TABLE I

H_2S-Formation by P. vulgaris from Various Sulfur-Containing Compounds

Compounds	H_2S tested by Pb-acetate qualitatively*				H_2S	
	1 hr.	2 hrs.	15 hrs.	24 hrf.	24 hrs.	72 hrs.
Control	—	—	—	—	2 μg.	2 μg.
L-Cysteine-HCl	##	##	##	##	43	
L-Cystine	##	##	##	##	53	
L-α-Dihydroxy-β-dithiodipropionic acid	—	—	±	+		15
β-Dithiodipropionic acid	—	—	—	—	2	2
N-Benzoyl-L-cystine	—	—	—	—	2	2
Dithiodiglycolic acid	—	—	—	—	2	2
Na thioglycolate	—	—	—	—	2	2
L-Cysteic acid	—	—	—	—	2	2
Taurin	—	—	—	—	2	2

* Hydrogen sulfide evolved was represented by the intensity of the color development of lead sulfide.

*II. Cysteinase**:

Preparation of Cells—Stock cultures of *E. coli* (the departmental collection of Osaka University) were grown on agar-plates. The cells after being harvested by centrifugation were resuspended in a medium consisting of 0.5 per cent peptone, 1.0 per cent broth and 0.1 per cent cysteine-HCl, and incubated at 37° for 2 hours on a shaking machine. They were reharvested by centrifugation and washed with distilled water. The paste of packed cells thus obtained was spread on a plate and dried under vacuum at −5°.

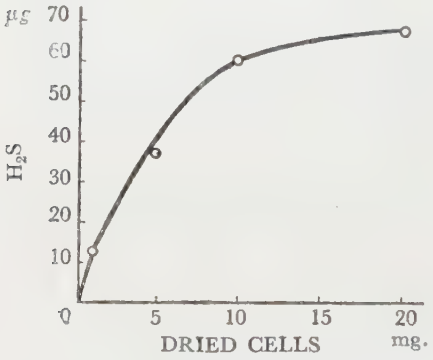


FIG. 1. Relationship of H_2S production to concentrotion of dried cells.

Total volume was 3 ml. containing 1 ml. of cysteine-HCl (5 mg.), 1 ml. of *M*/3 phosphate buffer (pH 6.4), dried cells mentioned above, and incubated for 1 hour at 37°.

Measurement of Hydrogen Sulfide—The usual assay was carried out by a slight modification of Porter's method (*21*). After the enzyme reaction was stopped by the addition of 2 ml. of 1 *N* H_2SO_4, the hydrogen sulfide evolved was absorbed in 0.6 per cent, zinc acetate, using a glass-stoppered bottle epuipped with a system for aeration. This was followed by the addition of *N*/100 iodine solution and 1 ml. of 1 *N* H_2SO_4, and subsequent titration with *N*/100 $Na_2S_2O_3$ solution.

Activity of Dried Cells—The activity of the dried cell preparation was proportional to the cell concentration, saturation being reached in the range of about 10 mg. (Fig. 1).

Inhibition of H_2S Production by Various Reagents—This was studied, using KCl, semicarbazide, and monoiodoacetate (Table II). These data showing the inhibitory action of carbonyl-reagents on cysteinase were also observed by Kallio and Porter (*21*) and by other research workers (*22*, *23*, *24*). In this connection, tryptophanase, β-tyrosinase and methioninase were also found to be inhibited by these reagents both in our laboratory and by others (*10*, *11*, *12*).

* This communication was presented at the Committee Meeting of the Enzymic Research of the Department of Education, on March 30, 1951.

TABLE II

Percentage of Inhibition of Cysteinase by Various Reagents

Inhibitors	Inhibition
	per cent
10^{-3} *M* Potassium cyanide	95
10^{-3} *M* Semicarbazide	92
10^{-2} *M* Monoidoacetate	100

Total volume was 3 ml. containing 1 ml. of cystine-HCl solution (5 mg.), 1 ml. of *M*/3 phosphate-buffer (pH 6.4) and 1 ml. of the dried cell suspension (10 mg.), inhibitors were as shown above, and incubation was 1 hours at 37°.

Fractional Precipitation of Cysteinase with Ammonium Sulfate—Fractions up to 50 per cent saturation with ammonium sulfate showed little activity compared to those of 60 to 100 per cent saturation. Fractions of 60 to 100 per cent saturation are shown in Fig. 2.

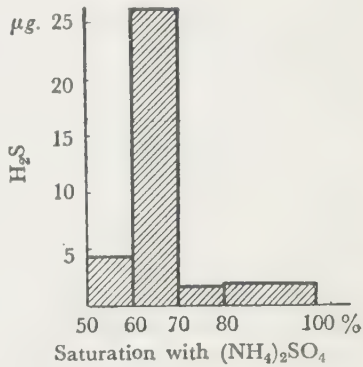


FIG. 2. The activity of each fraction of 60 to 100 per cent saturation with ammonium sulfate.

Total volume was 3 ml. containing 1 ml. of *M*/3 phosphate buffer (pH 6.4), 1 ml. of cysteine-HCl (5 mg.), 1 ml. of the enzyme preparation incubated for 1 hour at 37°.

Purification of the Enzyme—Dried cells were ground with 5 times their weight of quartz-sand in a mortar, suspended in distilled water (50 mg. per ml.) and placed in a refrigerator for 24 hours and allowed to freeze. The suspension was then brought to 37° in a water bath, and autolysis was allowed to proceed for 2 hours. The autolysate obtained by centrifugation was brough to 60 per cent saturation with ammonium sulfate. The precipitate which formed was separated and solid ammonium sulfate was added to the suppernatant layer to adjust the concentration to 100 per cent. The subsequent precipitate was dissolved in a minimum amount of chilled distilled water, and fractioned to 70 per cent saturation with ammonium sulfate. The precipitate which formed subsepuently was again dissolved in chilled water, and the solution was centrifuged, and absorbed on kaolin (10 per

cent) for 30 minutes at 0° at pH 5.5. The absorbant was washed with chilled distilled water until all traces of ammonium ion were removed and was then eluted with $M/10$ phosphate-buffer (pH 8.5) for 15 hours at 0°. The eluate was used for the following assays.

Activity of the Purified Enzyme—One ml. of the enzyme showed the maximumum H_2S production in the cysteine substrate as shown in Fig. 3.

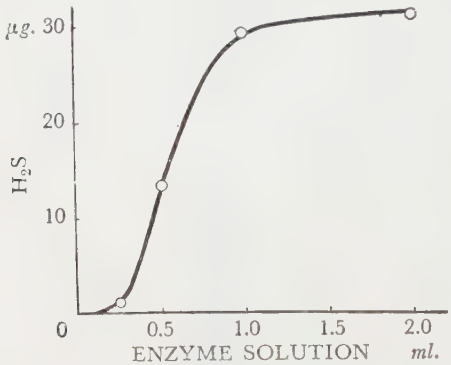


FIG. 3. Concentration curve of the purified enzyme.

Total volume was 3 ml, containing cysteine-HCl solution (5 mg.), 1 ml. of $M/3$ phosphate buffer (pH 6.4), and the enzyme solution as shown in the figure, incubated for 1 hour at 37°.

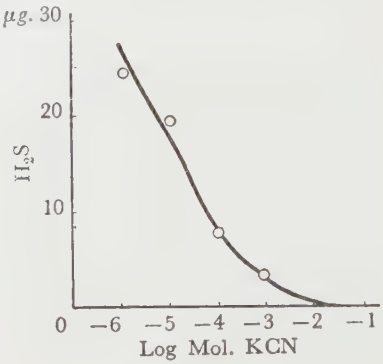


FIG. 4. Inhibition by KCN.

Total volume of 3 ml., containing 1 ml. of the enzyme solution, 1 ml. of cysteine-HCl solution (5 mg.) and 1 ml. of $M/3$ phosphate buffer (pH 6.4), incubated for 1 hour at 37°.

Inhibition of H_2S Production by KCN—The rate of the inhibition of hydrogen sulfide production by KCN added to the purified enzyme was found to decrease rapidly at concentrations of less than 10^{-3} M (Fig. 4).

Resolution of the Apoenzyme and Its Restoration by Pyridoxal and ATP—During the course of the purification process, $M/10$ KCN was added at the stage of 100 per cent saturation with solid ammonium sulfate and the final concentration of KCN adjusted to 10^{-3} M. The procedure of purification was otherwise unchanged. The enzyme thus obtained was completely resolved and regained its activity when supplied with pyridoxal and ATP* (Table III).

* More recently, the authors observed that the purified and resolved desulfhydrase (cysteinease) obtained from rat liver regained its activity by the addition of both pyridoxal and ATP.

TABLE III

Restroation of Activity of the Resolved Enzyme by the Addition of Pyridoxal and ATP

	None	ATP	Pyridoxal	Pyridoxal and ATP
H_2S produced $\mu g.$	2.0	3.9	2.7	19.7

Total volume was 3 ml., containing 1 ml., of the resolved enzyme, 1 ml. of cysteine-HCl solution (5 mg.) and 1 ml. of *M*/3 phosphate.buffer (pH 6.4), and wasin cubated for 1 hour at 37°. Pyridoxal-HCl: 500 $\mu g.$ (synthesized by Harris' method (*25*)). ATP: 1 mg. (Ba salt isolated from the rabbit muscle (*26*)).

Saturation Curve of Pyridoxal and ATP—The concentration of pyridoxal showed a saturation level of approximately 200 $\mu g.$ in regards to restoration of the activity of the resolved enzyme (Fig. 5).

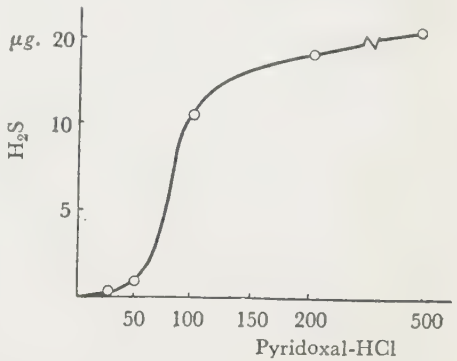


FIG. 5. The saturation curve of pyridoxal and ATP.

Under the same conditions as shown in Table III, in the precence of 1 mg. of ATP.

Study of Metabolites—

(a) *Alanine*—Each 1 ml. of the resolved enzyme solution, cysteine-HCl solution (total 5 mg.), *M*/3 phosphate buffer (pH 6.4) and 500 $\mu g.$ of pyridoxal-HCl and 1 mg. of ATP were placed in a tube, and incubated for 1 hour at 37°. After the reaction, mixture was deproteinized with alcohol, the alcoholic solution was concentrated under vacuum, and was tested by paper-chromatography, following the ascent technic, using phenol as the developing solution. Alanine was proved to be one of the metabolites by the ninhydrin test. No detection of serine was noted.

Since alanine was proved to be one of the metabolites in the degradation of cysteine as shown above, investigations were carried out manometrically using the conventional Warburg's apparatus to determine whether the partially purified enzyme (no KCN in this case) contained a system action on alanine.

The contents of the Warburg's vessels were as follows: Main compartment—1 ml. of *M*/3 phosphate buffer (pH 7.2), 1 ml. of the enzyme solution; Side arm—0.2 ml. of L-alanine (2 μM); Center well—0.1 ml. of saturated KOH.

No oxygen uptake was noticed, a fact indicating the absence of L-alanine deaminase. It is suggested that the latter may be inactivated by the process of fractional precipitations of the enzyme with ammonium sulfate. As observed by Green *et al.* (*27*), ammonium ion has been shown to inactivate L-alanine deaminase.

(b) *Ammonia*—The reaction mixture, containing each 1 ml. of the enzyme solution, 1 ml. of *M*/3 phosphate buffer (pH 6.4), and cysteine-HCl solution (5 mg.) was acidified by the addition of 2 ml. of 1 *N* H_2SO_4 (adjusted to pH 2) after an hour of incubation at 37°, and filtered. The filtrate was treated according to Archibald's method (*28*), and nesslerized ammonia was determined colorimetrically. Liberation of ammonia was not found, despite the production of hpdrogen sulfide (25 μg. in this case).

(c) *Pyruvate*—Pyruvate was determined by the method described by Friedmann *et al.* (*29*). The reaction mixture containing 1 ml. of enzyme solution, 1 ml. of *M*/3 phosphate buffere (pH 6.4) and 1 ml. of cysteine-HCl solution (solutions of 0.5 mg. and 5 mg. of cysteine, respectively) was incubated for 1 hour at 37°. The reaction process was stopped by the addition of 5 volumes of 10 per cent trichloracetic acid. Three ml. of the filtrate were treated with 2, 4-dinitrophenyl-hydrazine, and the color developed was determined by the use of Beckmann's spectrophotometer at 5200 Å (Table IV).

TABLE IV

Pyruvate Produced by the Purified Enzyme in 1 Hour at 37°

Cysteine-HCl	Pyruvate produced	H_2S produced
mg.	*μg.*	*μg.*
5	0	26
0.5	10	2

Pyruvate was detectable in the lower concentration of cysteine HCl (0.5 mg.), but not in its relatively higher concentration (5 mg,). The significance of this finding is discussed below,

Discussion

In view of the above experiments on the various derivatives of cysteine, it can be seen that hydrogen sulfide is more easily produced from either cysteine or cystine than with L-α-hydroxycarboxylic acid (a derivative of cystine). Hydrogen sulfide is not produced from the derivatives tested in Table I. In regads to the production of hydrogen sulfide from L-α-hydroxycarboxylic acid, it is believed that the source might possibly be cysteine rather than the acid, *per se*; that is, cysteine is formed bilogically during the incubation period from L-α-hydroxycarboxylic acid, as is noticed in a comparative sense in the case of phenol (*8*) and indole (*30*) from the L-α-alcoholic acids of tyrosine and tryptophan, Consequently, it might be postulated that hydrogen sulfide is evolved by the primary fission of the side chain of cysteine, without successive and stepwise degradation of the latter. This is shown schematically below.

In addition, it has been more recently recognized that hydrogen sulfide is produced after cystine is converted to cysteine (*21*). Furthermore, it was observed by Fromageot *et al.* (*31*) that the decomposition of homocystine (homocysteine) by mammalian livers produced hydrogen sulfide, and that this enzyme was independent of cysteinase.

According to our observations on the properties of the enzyme and the restoration of the resolved enzyme by the addition of pyridoxal and ATP, it may be assumed that these substances can be reconstructed and act as a coenzyme for cysteinase, Porter *et al.* (*21*), moreover, observed that various vitamins, and ions such as Mg^{++}, Mn^{++}, Co^{++}, and Zn^{++}, including their combinations had no effect on the activity of cysteinase with reference to hydrogen sulfide production. On the other hand, the reaction, such as indole formation, phenol formation ahd methylmercaptan formation were conceivably carried out by the same mechanism postulated herin, namely, by primary fission of the side chain (*10*, *11*, *12*). Since pyridoxal-phosphate is considered to be a coenzyme for the enzymatic production of phenol indole and methylmercaptan, it is postulated that this should also be the case with reference to cysteinase. Chargaff *et al.* (*32*), and Binkley (*33*) suggested that cysteinase was identical with enolase and serine-dehydrase, but Wood and Gunsalus (*34*), and Porter (*21*) have pointed out that it is absolutely independent of them.

Fromageot *et al.* (*2*) noticed the evolution of ammonia at time when hydrogen sulfide was produced. On the other hand, Takeda (*35*)

and Kiho (*36*) noted that the production of hydrogen sulfide was preceded by the deamination of cysteine by using the crude enzyme or living cells, In our studies using the purified enzyme, however, ammonia was not detected when the concentration of cysteine was 5 mg., yet alanine was dedected as one of the metabolites in this case. This fact might show that there is no interrelationship between deamination and desulfuration of cysteine by cysteinase*, The amino-group of cysteine can be assumed, to have a much closer affinity for the enzyme too, as regards to the fact that cysteine and cystine are sole sources of hydrogen sulfide. More recently, Dale *et al.* (*37*) elucidated the evidence that hydrogen sulfide, but not ammonia, was produced from cysteine by x-ray irradiation, while other amino acids prodced ammonia by the same process.

If hydrogen sulfide is liberated by the primary fission of cysteine, it may be presumed that besides amino acrylic acid either serine or alanine may be produced. Alanine, but not serine was detected in our experiments with paper-chromatography. Amino acrylic acid and alanine can not be distinguished from one another with this method, but the former is probably converted to pyruvate and ammonia. The data as shown in Table IV, demonstrate that alanine is detected in the higher concentration of cysteine, and that pyruvate is observed in the lower level, indicating the possibility that in the former case, the SH-group of cysteine reduces amino acrylic acid to alanine, oxidizing itself to cystine.

In lower concentration of cysteine:

$$SH\text{-}CH_2\text{-}CHNH_2\text{-}COOH \longrightarrow H_2S + H_2C{=}CNH_2\text{-}COOH \qquad \text{Ep. (1)}$$

$$H_2C{=}CNH_2\text{-}COOH \longrightarrow H_3C\text{-}C(=NH)\text{-}COOH \xrightarrow{+H_2O} H_3C\text{-}CO\text{-}COOH + NH_3 \qquad \text{Ep, (2)}$$

In higher concentration of cysteine:

$$2\,SH\text{-}CH_2\text{-}CHNH_2\text{-}COOH + H_2C{=}CNH_2\text{-}COOH \longrightarrow (\text{-}S\text{-}CH_2NH_3\text{-}COOH)_2 + H_2C\text{-}CHNH_2\text{-}COOH \qquad \text{Ep. (3)}$$

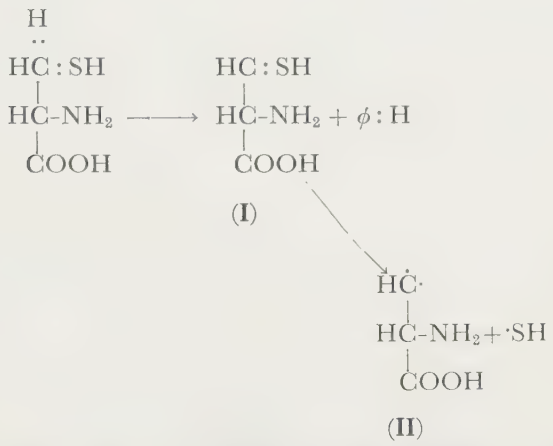
It can easily be seen that pyruvate is not the product of the deamination of alanine produced by way of the reaction of Ep. (3) on the basis of the evidence that there is no system which attacks alanine in the purified

* In this connection, it was observed in Obata Laboratory of the Hokkaido University that a certain fungus grown on cysteine did not produce hydrogen sulfide, despite the fact that cysteine was being utilized as a source of nitrogen. In this fungus growth, there is not interrelationship between nitrogen utilization and hydrogen sulfide production.

enzyme. The reaction of Ep. (2) process non-enzymatically.

The above assumption would be more appropriate in the case of cysteinase, but not for indole and phenol formations, in the light of our studies on the mechanism of the formations of indole, phenol, methylmercaptan and hydrogen sulfide. In fact, data on tryptophanase by Happold (*38*) and Suzuki (*39*) show that even in this case, alanine is detected in the higher concentration of the tryptophan as substrate, and, on the contrary not alanine, but pyruvate in its lower level. The authors observed that amino butyric acid was produced in the higher concentration of methionine by methioninase (*12*). Therefore, it would be more plausible to explain the mechanism of cysteinase according to that of indole formation by Happold *et al.* (*40*) stated below, instead of through the above mechaism.

If the enzyme is represented as ϕ(R-CHO) (R-CHO indicating the active group, for instance pyridoxal) with its free radical, (R-:$\dot{C}$H:O) (ϕ), the primary fission can be assumed to proceed as follows, together with the intermediary product as its mesomeric type (**I**) followed by the fission between the C-S bond:



The free radical ·SH would propagate the reaction to produce (**I**) after forming hydrogen sulfide. As (**II**) is equal to amino acrylic acid, it would be converted to alanine in the final stage by ϕ:H, presumably to a greater extent by the higher concentration of cysteine, despite the fact that it is decomposed non-enzymatically to form pyruvate and ammonia in lower concentrations. Assuming the mesomeric type of

the reactive group of the enzyme to be as shown in $R\text{-}\underset{H}{\overset{\cdot\cdot}{C}}O \overset{+H}{\underset{-H}{\rightleftharpoons}} R\text{-}:\underset{\cdot\cdot}{\overset{\cdot\cdot}{C}}O:H$, the mechanism availing for the other enzymes might be suggested on the basis of the dehydrogenation.

SUMMARY

1. It was observed that hydrogen sulfide was praduced only from L-cysteine, L-cystine, and L-α-hydroxycarboxylic acid by using *Proteus vulgaris*, but not from N-benzoyl-L-cystine, β-dithiopropionic acid, dithiodiglycolic acid, Na thioglycolate, L-cysteic acid or taurin. It is postulated that this production does not go through successive degradation of the side chain, but through primary fission.

2. Cysteinase was obtained in the cell-free state from toe cultures of *E. coli* adapted to cysteine.

3. The activity of the enzyme was inhibited by carbonyl-reagents, such as KCN and semicarbazide.

4. Purification and resolution of cysteinase were described.

5. The resolved enzyme regained its activity when supplied with both pyridoxal and ATP as its coenzyme.

6. Alanine wai demonstrated to be formed in the higher concentration of cysteine, instead of pyruvate and ammonia. On the contray, pyruvate was detectable in its lower level.

7. The mechanism of cysteinase was discussed on the basis of the data obtained.

Appendix:—More recently, it was observed by Kallio (*41*) that the desulfhydrase system obtained from *Proteus morganii* was activated by the addition of pyridoxal.phosphate with the production of hydrogen sulfide, ammonia and α-keto acid.

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PYTHCHOLIC LACTONE AND 3, 12-DIHYDROXY-7-KETOCHOLANIC ACID FROM THE BILE OF BOIE (*Python reticulatus*)

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From the bile of boile, the authors have found 3, 12-dihydroxy-7-ketocholanic acid (m. p., 172-174°) and pythcholic lactone (m. p., 263-264°; ketolactone, 244-245° and dioxime, 198-200°). The latter has been already isolated by Haslewood (*1*) from the same bile. The former acid seems to be rather an intermediate metabolite from cholic acid to desoxycholic acid.

EXPERIMENTAL

About 80 ml. of dark brown bile of boie were separated from mucin by adding alcohol, hydrolyzed in 10% pottasium hydroxide solution for 30 hours, and acidified with dilute hydrochloric acid. The precipitate was filtered and referred to as (A); while the filtrate as (B).

By dissolving the precipitate (A) in 10% sodium carbonate solution, the insoluble matter (C) was separated.

The soda solution was acidified with dilute hydrochloric acid, extracted with ether, and the ether extract was further extracted with 15% hydrochloric acid. The hydrochloric acid extract was neutralized to the point just acidic to congo red with soda, and again extracted with ether. The ether layer was washed with water, dried and evaporated to dryness. The residue was crystallized from dilute alcohol, giving 50 mg. of needles, (m.p. at 172-174°). Thus obtained crystals gave no melting point depression when mixed with the authentic sample of 3, 12-dihydroxy-7-ketocholanic acid.

2. 080 mg. of substance gave 1.750 mg. of H_2O and 5.450 mg. of CO_2.

Calcd. for $C_{24}H_{38}O_5$	C 70.88%; H 9.42%
Found	C 71.50%; H 9.42%

It was methylated with diazomethane and crystallized from dilute

methanol. Melting point and mixed melting point with methyl 3, 12-dihydroxy-7-ketolanate were the same (157-162°).

3.900 mg. of substance gave 3.310 mg. of H_2O and 10.230 mg. of CO_2.
3.790 mg. of substance gave 3.280 mg. of H_2O and 9.920 mg. of CO_2.

Calcd. for $C_{25}H_{40}O_5$	C 71.38% ;	H 9.37%
Found	C 71.48% ;	H 9.50%
	C 71.48% ;	H 9.68%

The methylester monoxime decomposed at 176°.

3.193 mg. of substance gave 0.118 ml. of N_2-gas (128.8°, 750.1 mmHg.)

Calcd. for $C_{25}H_{41}O_5N$	N 3.219%
Found	3.15%

Filtrate B was made alkaline with soda, evaporated to dryness and extracted with alcohol. Alcohol extract was again evaporated and the residue was crystallized from dilute alcohol (needles; m.p., 263-264°).

The same product was gained from fraction C.

The total yield of the product was 150 mg. It formed no precipitation with digitonin and was soluble in warm caustic soda solution.

4.430 mg. of substance gave 4.080 mg. of H_2O and 12.000 mg. of CO_2

Calcd. for $C_{24}H_{38}O_4$	C 73.78% ;	H 9.81%
Found	C 73.92% ;	H 10.32%

Twenty mg. of the lactone were oxidized with chromic acid in acetic acid solution, and 12 mg. of ketolactone were formed. It formed needles from dilute methanol, melted at 244-245° and gave positive Jaffe's reaction.

3.390 mg. of substance gave 9.220 mg. of CO_2 and 2.600 mg. of H_2O

Calcd. for $C_{24}H_{34}O_4$	C 74.57% ;	H 8.86%
Found	C 74.22% ;	H 8.57%

Dioxime lactone derived from ketolactone decomposed at 198-200°.

2.727 mg. of substance gave 0.167 ml. N_2-gas (29.2°, 749.7 mm.Hg)

Calcd. for $C_{24}H_{36}O_4N_2$	N 6.72%
Found	N 6.83%

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